

Sharing research tools is a tradition worth defending

Increasingly tough conditions being attached to the transfer of experimental tools between researchers are threatening science's tradition of open communication. Agreement on optimal terms would help all sides.

Isaac Newton neatly encapsulated the cumulative and communal nature of the scientific enterprise when he claimed to have seen further by standing on the shoulders of giants — building on ideas and mathematical tools that had been described in his colleagues' letters or publications. For today's theorists, not much has changed: a published paper typically contains all the information necessary for others to reproduce and extend the author's work. But modern experimental science can also depend on research tools that may have taken months or years to develop — the atom traps that enable Bose–Einstein condensation, for example, or the huge and intricate detectors that capture data at particle accelerators.

In most areas of physical science, research tools that have been painstakingly developed cannot be easily reproduced; thus the scientist who has developed such a tool has a significant head-start on potential competitors. But in much of biology, thanks to techniques such as cloning and the polymerase chain reaction, tools that are difficult to produce can be trivial to replicate. Such is the case with the bacterial DNA molecules known as plasmids, which are used to create genetically engineered cells and organisms.

The existence of such unique but easily propagatable research tools has meant that sharing materials has become the norm among biologists. The National Institutes of Health (NIH) includes among the terms and conditions of its awards a statement that unique research resources should be made available when their existence has been reported in the literature. Many journals, including *Nature*, require authors to promise to share materials for academic use.

So far, so good. But there is increasing concern in the biomedical community that some researchers are prepared to share their materials only when doing so does not threaten their competitive advantage. And in cases — ever more frequent — where there is a prospect of commercialization, the terms dictated for sharing can

be unreasonably difficult to meet (see *Nature* 393, 499; 1998).

Journals have an interest in ensuring that the research they publish can be reproduced; this — and a broader interest in promoting the rapid progress of scientific enquiry — is why *Nature* requires authors to share materials. But neither of these aims is served by requiring a vulnerable postdoc to give materials to a large, well-funded competitor who intends to race the postdoc to the next obvious result.

One end of the spectrum of opinion on this question has been enunciated by the Society for Neuroscience, whose council has just approved 35 pages of guidelines for “responsible conduct” in scientific communication (see <http://www.sfn.org/guidelines/>). These advise that materials should be provided “without restrictions” — and allow for a delay only in cases of compounds being developed as therapeutic agents. The ‘teeth’ are provided by the editors of *The Journal of Neuroscience*, who are in a position to reject papers by those who do not comply, and by the society's ability to bar malefactors from presenting results at its annual meeting. It remains to be seen whether these teeth will be sharp enough to change behaviour.

The society is to be applauded for enunciating a clear policy with accompanying sanctions. But its bold stance against any restrictions on transferred materials seems wishful thinking, given the growing use of highly restrictive materials transfer agreements (MTAs) by companies and universities alike. Too often, the negotiation of these agreements is the rate-limiting step in sharing materials. One *Nature* reader has been waiting six months to receive an MTA — and fears that when it finally arrives, his funding agency will not allow him to sign it.

The NIH's working group on research tools (see *Nature* 393, 505; 1998) has recommended the widespread adoption of a uniform MTA. If universities, companies and funding agencies were to agree on such a form, journal editors could require that it be used by their authors. No one need lose but the lawyers. □

Time to lift embryo research ban

Last week's announcement on human embryo stem cells requires a change in the US law on embryo research.

One of the consistent complaints about modern science is that it often progresses faster than society's ability to ensure that its potential applications are properly regulated. Frequently it is the scientific community and its industrial counterparts who are seen as responsible for this state of affairs. But in the case of last week's dramatic announcement of a major breakthrough in the culture of human embryonic stem cells (see page 104), political authorities — and in this case, the US Congress — have only themselves to blame. Maintaining a ban on such research with federal funds, while leaving the private sector totally unregulated, has ended up with the worst of both worlds.

Geron, the company that has negotiated licences to the research results in question, has made it clear that the use of the technology will be regulated — but, given the political vacuum, by them, not by any democratically representative body. Strict conditions — as are increas-

ingly required in other areas of biomedical research (see above) — will have to be met by anyone seeking to use the stem cells for research. Some of these ethical requirements will be desirable, but they should not be left in the hands of a commercial company.

If there was ever a good time to reopen the congressional debate on the ban on federally funded research on embryos, it must surely be now. It is important that the arguments of those in favour of such a ban are given due respect — and, furthermore, are not trampled into the dust as the public clamours for the therapeutic dividends that Geron's new techniques offer. But it is also important that the conditions under which the research and its applications develop are subject to the broadest public scrutiny and consistent regulation. In the current circumstances, that cannot happen. Changing the law to make this possible is the only sensible — and responsible — way forward. □



US court backs authors in fight over article reproduction rights

[SAN DIEGO] A copyright-infringement lawsuit against a company in Colorado that operates the world's largest index of journals may change how scientific articles are distributed electronically and give researchers newly defined control over their publications.

Five freelance authors who are suing The UnCover Co. won a federal court ruling last month under which the company can no longer distribute copies of a journal or magazine article unless it has a signed copyright release covering all authors.

The UnCover Co. retrieves articles from journals, copies them and charges a \$13 fee for their distribution — often, according to the allegations by the suing authors, without the full authority of all the authors. The company is owned by the London-based Dialog Corporation.

Until now, although the company may have received releases from publishers of the journals, it has not felt it necessary to obtain such a release from each author of a reproduced article. Under the judge's ruling, however, it must now do so — or the journal publishers themselves must have secured all future publication rights from the authors.

The ruling sets the stage for an era of negotiation between authors — whether scientists or freelance writers — and journal publishers over the rights to and income from article reproductions. Many journals, and the professional societies and companies

that publish them, now seek all copyright rights from authors.

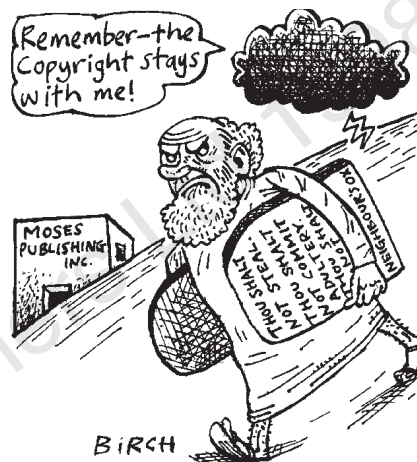
But scientists, who are looking more and more to the Internet to disseminate peer-reviewed research for rapid comment and use, are increasingly challenging this practice, seeking to retain future copyright rights.

"I think this is a very, very important issue," says Steven M. Bachrach, a theoretical organic chemist at Northern Illinois University who has written extensively about authors' rights. "Most of my colleagues are unaware of the copyright issue; this may make people more aware."

The ruling, handed down on 13 October in a US District Court in San Francisco by Judge Fern M. Smith, has been greeted enthusiastically by the suing authors and their attorneys, but with gloomy predictions by The UnCover Co.

"By the court's ruling, anyone who wants to copy portions of a collective work needs permission from the author and the publisher," says A. J. De Bartolomeo, an attorney who represents the authors. "You can't just get permission from the publisher, which is what The UnCover Co. claimed to be doing."

The company warns in a statement that the ruling "further restricts the dissemination of scholarly works by requiring that document delivery services obtain permission from both publisher and author before delivery of a work or research article". It adds: "The court acknowledged in its decision that



'the academic use of articles will be made more difficult' by this ruling."

But Daniel A. Reidy, another attorney who acted for the authors, argued that The UnCover Co. itself restricts scientific discourse by charging the price of a hard-cover book for a single article sent from a computerized database. "If anything, The UnCover Co. has a chilling effect by forcing people to pay huge fees for article delivery," he says.

There is much concern about the long-term implications of ownership and control of such databases. Reidy compares the growing centralization of power over databases to the railroad monopolies of a century ago: "Do you want commercial companies monopolizing academic articles?"

The suing authors — who write freelance articles about sports and sociology, and poetry — see the matter as one of misappropriation. "If you want to borrow my work or use it, call and ask me; don't take it without my permission and try to sell it," says James Tunney, a retired American football referee from Pebble Beach, California. "It is thievery."

Another author, Joan Ryan, a San Francisco journalist, says: "I don't care if a student doing a term paper uses my stuff, but if a company takes my material without compensation I scream bloody murder."

Ward Shaw, chief executive of The UnCover Co., says the company believes that the document deliveries "were made under good faith permission from the publishers". He says a \$3 copyright fee is paid to publishers whenever an article is distributed. But opposing attorneys questioned how often this is done, adding that authors of the copied articles usually received nothing.

Calling the court decision "onerous", Shaw describes the idea of getting copyright releases from all authors and the publisher as "unworkable". The UnCover Co. maintains a

Lifting the lid on a mediaeval mystery



[MUNICH] Italian scientists last week opened the sarcophagus of Frederick the Second (1194–1250), King of Sicily and Holy Roman Emperor, and took samples of dust, textiles, skin and bones under special light.

Scientists will analyse his DNA to establish lineage, and the microorganisms in the sarcophagus to determine why his body is so well preserved. They will also try to establish if Frederick died from poisoning, as suspected. Frederick, who founded the University of Naples, is seen by many as Europe's first experimental scientist.

The clean room in which the opening was carried out was donated by the German company Zander. It was built around the sarcophagus in Palermo cathedral, Sicily.

The sarcophagus was opened only 40 cm as a compromise with the Bishop of Palermo, who considered the endeavour disrespectful. The opening was shown live on Italian TV.

Alison Abbott

index of at least 8 million articles from 17,000 journals and magazines. It also has arrangements with more than 35 libraries to retrieve documents from their archives.

But the suing authors express little sympathy for Shaw's position. "If they can't figure out how to distribute material legally, they should get into another line of work," says Ryan. "People did research before the Internet. Rights and responsibilities need to keep up with technology."

Alexander Fowler, of the Electronic Frontier Foundation of San Francisco, agrees that rights of authors and Internet technology can collide. "It is unfortunate that we are still locked into a paper-based distribution model," says Fowler. "We have to buy reprints to disseminate ideas; we potentially infringe copyrights if we put our own articles on our own websites."

Working with the American Academy of Arts and Sciences, Fowler and Bachrach are among a dozen authors who recently published a paper in *Science* advocating that scientists should retain the rights to their articles after they are first published. This runs counter to the view of some journals, such as *Science* and *Nature*, whose publishers seek to retain copyright, arguing that value has been added by the selection and editing process.

The creators of The UnCover Co. clearly had a vision when the company was started five years ago in Denver by a group of academic librarians. Nearly 20 years ago, the librarians, headed by Shaw, created the Colorado Alliance of Research Libraries (CARL), a nonprofit organization designed to assist academic libraries in the state with inter-library transfers of publications.

As the computerized information age bloomed, the group was well positioned to expand and profit from new technologies. A decade ago, Shaw and colleagues left the nonprofit alliance and started the for-profit CARL Corporation, which then gave birth to The UnCover Co. Shaw and the other founders have since sold their stakes in CARL to a corporation.

Attorneys for The UnCover Co. are seeking to appeal against the judge's decision. The full ramifications of the ruling will not be known for some time, but it could set a major precedent in copyright law.

The lawsuit against The UnCover Co. is a class action, where the five suing authors are acting on behalf of any other authors whose copyrights may have been infringed. It seeks to recover damages for any authors who had their work distributed without permission during the three years before the filing of the lawsuit in October 1997.

At a hearing next week, attorneys for the authors will seek court approval for a fuller description of the class of authors to be included in the lawsuit. Both sides will then proceed with preparations for a future trial to determine damages.

Rex Dalton

UK initiatives aim to boost high-tech investment

[LONDON] The British government has announced a series of measures to help universities overcome the barriers to commercializing the results of their scientific research. The most concrete is a £25 million (US\$41 million) grant to create between six and eight 'Institutes of Enterprise' attached to universities.

The measures are the latest — and perhaps the most concerted — attempts to address the widely-held concern that Britain lags behind its competitors in turning high quality research into commercial success. The new institutes, for example, will teach entrepreneurship and business skills, and provide university start-up companies with expert financial analysis.

The measures were announced last week by Britain's Chancellor of the Exchequer Gordon Brown in a report outlining the thinking behind the forthcoming budget.

Brown announced that the government is considering offering a tax credit for small and medium sized companies yet to make a profit. The size of the credit will depend on the volume of research and development expenditure, and is designed to stop these companies cutting back on R&D spending as they struggle to pay taxes.

The government will also announce in the budget whether it will give tax incentives on share ownership to key managers of small high technology companies. This would allow technology start-up companies to attract and retain top managers.

Both moves were recommended by a government-appointed committee of representatives of high technology companies and the investment community, which looked at ways of improving the financing of high technology businesses. The committee, chaired by Sir Peter Williams, chairman of the scientific instrumentation company Oxford Instruments, published its report last week.

The Williams committee is among the many groups — including universities, trades unions, and the pressure group Save British Science — to have welcomed the government's measures. But Williams himself says more needs to be done if the government is serious about gaining greater economic benefit from research.

The committee found that a key difficulty is the British reluctance to invest in new high technology companies at the start-up stage. Last year, total UK high technology venture capital amounted to £349 million, compared with £5.8 billion in the United States.

Only 5 per cent of British venture capital goes to start up companies, which is less than

in the United States. Similarly, in the United States, 5 per cent of pension fund assets are invested in venture capital. But in the United Kingdom, pension funds, the largest investors in the Stock Exchange, contribute just 0.75 per cent. More than half of the venture capital invested in British companies comes from overseas.

Investment decisions — including those for long-term finance such as pension funds — are often based on short term performance statistics. One reason for the lack of high technology venture capital from British insurance companies and pension funds, says Williams, is an outdated perception that early stage technology venture capital is too risky in the short term.

The Williams report quotes data from the British Venture Capital Association showing that early stage companies have been providing an average 22.9 per cent



Williams: keen to see more tax incentives.

annual return on investment for the past three years, comparable to the performance of the top 100 companies listed on the London Stock Exchange.

More money might flow into early-stage technology companies, the committee believes, if the government sets up special investment trusts exempt from capital gains tax that consist only of new high technology companies. Big investment institutions and individuals should be allowed to invest.

The relatively large tax burden on investors also limits investment in new technology companies, says Williams. His committee believes that a lowering of capital gains tax would lead to a rise in venture capital investment, as it did in the United States when taxes were lowered in 1982.

The committee wants the government to exempt from capital gains tax investors who have held stocks in newly-established high technology companies for five years. But Treasury officials say this is unlikely to be in the next budget, as the government relaxed capital gains tax only last year.

The Williams committee also highlighted communication difficulties between technology companies and investors as a barrier to the commercialization of research. Investors should understand that the results of life science research can take up to 15 years to reach the market.

Ehsan Masood

Private observatories seek public funding

[WASHINGTON] Privately run US observatories possessing high-performance telescopes but not the funds to equip them with state-of-the-art instruments have come up with a plan to persuade the National Science Foundation (NSF) to help pay for instrumentation.

The directors of the top observatories, which include the McDonald Observatory in Texas, the Steward Observatory in Arizona and the Harvard Smithsonian Center for Astrophysics, have suggested that the NSF should provide up to \$8 million a year in matching funds to pay for the instruments.

The plan has been agreed by a coordinating council of the directors of these observatories and of the National Optical Astronomy Observatories (NOAO). The NOAO is run by a consortium for the NSF and would be allowed to compete with the private facilities for matching instrumentation funds. The plan would replace the Facilities Instrumentation Program, under which the NSF pays for instruments and the observatories in exchange make telescope time available to outside researchers.

This programme was proposed three years ago by a panel of the National Research Council (NRC) chaired by Richard McCray of the University of Colorado. But it is virtually moribund because the observatories are reluctant to release the time in exchange for NSF support. The observatories' alternative — called the Revised Facilities Instrumentation Program — was due to be considered at a meeting of the NRC's Committee on Astronomy and Astrophysics in Washington this Tuesday (10 November).

The directors hope that the committee,



Still in the line of sight: The Kitt Peak Observatory is currently the only facility in the United States with 4-metre-class telescopes that are open to all astronomers.

which acts as a *de facto* advisory board for the NSF astronomy programme, will endorse their proposal in time for its inclusion in the NSF's next budget proposal, for the year 2000, to be finalized in February.

But the committee is more likely to study the proposal for a couple of months and, according to an official close to the proposal, then to back a compromise allowing the NSF to fund instruments on the basis of contributions of time or cash, or a mixture of the two.

Joseph Miller, director of the Lick Observatory in California and chairman of the committee of observatory directors that created the proposal, says the instrumentation is needed to keep US telescopes competitive with others, such as the European Southern Observatory in Chile. He denies that the plan could be construed as self-serving. "We have a \$200-million facility in Hawaii, which didn't cost the NSF a cent, but for \$2 million

they could vastly increase its [power]," he says, referring to the Keck telescopes.

But the revised proposal would clearly neglect a primary objective of the McCray panel, namely, to gain access to the best private telescopes for researchers from other institutions. At present they must depend on NOAO's ageing array of optical telescopes at Kitt Peak in Arizona. When the McCray panel reported in 1995, it was widely expected that NOAO would close several telescopes because of expected cuts in the NSF's overall budget. The budget has since grown, and most of the telescopes are still operating.

Still, supporters of the McCray proposal were expected to tell the NRC committee this week that the original idea of getting the observatories to free up time for outside researchers in exchange for NSF support remains sound. They admit it hasn't worked so far — only two small instruments have been supported by the NSF, and no more are pending. But they say that lukewarm support from the agency, combined with what appears to have been a tacit agreement among some observatory directors not to apply for the support on the available terms, have killed interest in it.

Several directors, for their part, remain adamantly opposed to the idea of swapping time for NSF support. They fear a 'slippery slope' by which the NSF would start to ask for telescope time in exchange for other grants. They also argue that releasing time to the federal agency would anger the founders who paid for the telescopes.

The disagreement reflects a history of bad blood between the 'haves' and the 'have-nots' in US optical astronomy. "The NSF spends two-thirds of its astronomy budget on the national observatories — but many of us feel that the grants programme is really the heart of the discipline," says Miller. The grants programme supports many of the top astronomers and astrophysicists using the private facilities.

Colin MacLiwain

Changes in store for Japan's vet colleges

[TOKYO] In a bid to improve the falling standard of veterinary education in Japan, the association representing veterinary schools has proposed a drastic reorganization of university veterinary colleges.

The association suggests that veterinary colleges at eight national universities be merged to create two schools at Kyushu University in southern Japan and Tohoku University in the north. These would each absorb four existing departments, leaving Tokyo and Hokkaido as the only other national universities with veterinary colleges. Plans released last month say the mergers should be completed by 2001.

Hideaki Karaki, president of the association and professor of veterinary pharmacology at Tokyo University, says that "a shortage of staff makes it difficult to train good clinicians", although the existing courses provide good

training in basic research.

The broadening of veterinary sciences, caused by the emergence of prion diseases and other cross-species transmission, has provoked concern over the poor standard of veterinary scientists in Japan. "There is a great demand for highly trained researchers and clinicians in areas such as public health and virology," says Karaki. "The proposed merger should create an integrated system to provide balanced education in preclinical and clinical veterinary medicine."

But the closures of veterinary colleges are likely to affect local agricultural industries, and may face strong local opposition. The government says that applications to veterinary colleges at national universities have trebled in the past decade — and that the prospect of even greater competition is likely to make the proposed changes unpopular.

Asako Saegusa

Italian charity offers an alternative to the *concorsi*

[MUNICH] In the first major challenge to the centralized selection and funding of academic positions in Italy, an Italian medical research charity that raises money through a television show is to launch a series of high-level research positions.

Telethon, which predominantly funds research in genetic diseases, will put out a 12-billion-lire (US\$7.3-million) call for proposals next spring for one so-called Telethon scientist (equivalent to a full university professor), two associate Telethon scientists and seven assistant Telethon scientists. A similar call for proposals is planned each year, provided that the money raised through the annual television show continues to rise. This year Telethon raised a record IL37 billion.

Academic positions in Italian universities and public research institutes are, by law, filled through national competitions called *concorsi*. However, the *concorsi* system has been increasingly criticized for allowing personal contacts with members of the selection committees to influence some appointment decisions (see *Nature* 378, 228; 1995).

Telethon-funded posts will be selected by the charity's international scientific advisory board, which decides on the distribution of all Telethon research funds. Recent changes in the rules governing university administration have made it possible to avoid the *concorsi* system.

Each position will last for five years, and will come with generous project funding, as well as a salary nearly one-third higher than

that offered by equivalent publicly funded positions. Applicants must secure the agreement of a university department or national research institute to act as their host.

The charity hopes that a Telethon post will be considered as prestigious as that of a Howard Hughes investigator, the model on which it is based. But not all scientists are convinced that the Hughes model can be successfully transferred to the status-conscious Italian academic culture.

Piergiorgio Strata, for example, professor of neuroscience at the University of Turin and a member of Telethon's scientific advisory committee, is a strong supporter of the Telethon posts. But he worries that, despite the money and research freedom they offer, Telethon scientists may be seen as less prestigious than conventional university professors.

Andrea Ballabio, however, director of the Milan-based Telethon Institute of Genetics and Medicine (Tigem), says the Telethon initiative is needed to promote research excellence in Italy and to give talented researchers working abroad a way of returning home independently of personal contacts.

"The *concorsi* system is very bad and it has been difficult to change from the inside," he says. "It is right to try to change it through example from the outside, as Telethon is doing." It is especially important, he says, that the posts will be selected by an international committee "who won't care about Italian political games and will make judgements from a purely scientific standpoint". **Alison Abbott**

India agrees to conduct AIDS vaccine trials

[NEW DELHI] After years of hesitation, India has joined the global efforts to develop an AIDS vaccine by deciding to conduct human trials with vaccines developed either indigenously or with international collaboration. The decision, which still requires cabinet approval, was taken last Sunday (8 November) after a meeting of officials from India's National AIDS Control Organization (NACO), the US National Institutes of Health (NIH) and the United Nations AIDS programme.

J. V. R. Prasada Rao, chief of NACO, announced that a core group will be formed to work out the programme's details. With assistance from NIH and the United Nations, NACO plans to set up a multi-disciplinary team to take up all aspects of AIDS vaccine research and trials.

"It will link Indian AIDS researchers with their counterparts abroad, and develop a flexible plan for producing and testing

candidate vaccines," says Vulimiri Ramalingaswami, former chief of Indian medical research, and prime mover of the vaccine initiative. Sanjay Mehendale, director of the National AIDS Research Institute in Pune, says India will be ready to launch the trials in two years.

The choice of vaccine — Indian or foreign — is a matter on which "we have to tread very carefully," says Rao. India backed out of international clinical trials nine years ago to avoid criticism that its people were being used as guinea-pigs by foreign researchers.

Rao says the vaccine should be based on virus isolates from India, and should be made available at an affordable price. A vaccine manufactured abroad must undergo phase I trial in the country of origin before use.

Carol Heilman, deputy director of NIH's AIDS programme, says her agency will help by facilitating interaction between Indian researchers and US companies. **K. S. Jayaraman**

NASA is urged to use less home grown technology

[WASHINGTON] The US space agency NASA should look more to universities and industry, and less to its own centres, to develop advanced technologies for missions, says a report from the Space Studies Board of the National Academy of Sciences.

"NASA should recognize that, when a technology important to its missions is available on the outside from academic or industrial organizations, this represents a NASA success," says the report. It was written by a committee chaired by Daniel Fink, a former aerospace industry executive and deputy director for space research at the US defence department, who now works as a private consultant.

The panel was formed at NASA's request to assess technology development in the agency's Office of Space Science. It argues that the switch to cheaper space missions has made the agency reliant on inventions that reduce spacecraft weight (and therefore launch costs) without sacrificing performance. These developments range from miniaturized instruments to lighter propulsion, power and communication systems.

The panel applauds NASA's recent decision to give responsibility for much of this technology to the space science office. But it worries that the agency is hoarding too much development work for its own centres.

Less than half of NASA's technology development funds are open to non-NASA competitors. The agency is moving towards a congressionally mandated goal of 75 per cent, but the panel sees no reason to stop there.

In deciding whether to develop a technology in-house or farm out the work, each NASA centre considers a list of its "core competencies". But the list may not be realistic. "Some centres claim that their core competencies cover an extensive range of technologies," wrote Fink's committee. "No organization that has realistic fiscal constraints can hope to be competitive or world class across such a sweep." A centre that exaggerates its abilities "will be less likely to rely on other organizations where world-class capabilities actually reside".

The panel plays down the argument that agency engineers need to keep their hand in technology development to be smarter buyers. "An organization does not need to maintain hands-on efforts to make sound decisions about acquiring goods and services from outside sources."

The committee recommends that NASA centres trim their list of "core competencies" and subject the revised lists to outside peer review. **Tony Reichhardt**

Highbrow 'club' seeks the common touch

[LONDON] One of the few British scientists to enjoy a high media profile has taken on the daunting task of 'modernizing' one of the country's most distinguished centres for research and science communication: the Royal Institution.

The institution celebrates its bicentenary next year, and Susan Greenfield, its first woman director, plans to mark the occasion with major changes. Greenfield, professor of pharmacology at the University of Oxford, took up her job at the institution last month.

Greenfield says she is keen to take the institution's successful lecture-demonstrations to schools in Britain's deprived inner cities and to prisons to show how science can illustrate contemporary issues such as the dangers of drug abuse.

"I would like the Royal Institution to be perceived as a place that explains to all sectors of society — not just the middle classes — how the world is changing, why and what impact it will have on their lives," she says.

The institution was set up in 1799 by a group of well-connected scientists, and is housed in an impressive building off Piccadilly in central London. It has a research laboratory where 10 elements were discovered. Seven of its scientists have won Nobel prizes, including the father and son physicists Henry and Lawrence Bragg and the chemist George Porter.

The laboratory is now known as the Davy Faraday Research Laboratory after two of its early researchers, Humphry Davy and Michael Faraday. It still ranks among the world's top laboratories in solid-state chemistry, producing around 100 papers annually.

But the institution's founders had a second aim: to communicate the excitement of research to non-scientists. This was done by



All change: Greenfield (inset) wants to modernize the image of the Royal Institution's 'discourses'.

holding public lectures. Faraday also pioneered 'evening discourses' for members, and annual 'Christmas lectures for juveniles'. Despite the passage of time, the format and titles of many of the institution's activities remain largely unchanged. What are now known simply as 'Christmas Lectures' attract a television audience of two million, while 40,000 children visit the institution each year.

A degree of modernization was started seven years ago by Greenfield's predecessor, the chemist Peter Day, who helped put the finances back into the black — the institution has lost money for most of its history, and has come close to bankruptcy several times. Day organized the refurbishment of the building including its main lecture theatre.

Day encouraged the expansion of weekend 'master classes' around the country for mathematically able children, as well as an

outreach programme. Greenfield acknowledges that the "chattering classes" will remain one of the institution's target audiences. But she wants to change the organization's overall elitist image.

Another image she wants to replace is that of the scientist in the white coat — the traditional attire of Friday evening lecturers — "talking down" to people. Greenfield is keen to create a centre where the public, politicians, social scientists, and representatives of the arts and religious communities can meet and debate with scientists on equal terms.

"I'm not talking pedagogy here," she says. "People have fears about science. They don't want just to be told the facts. They need a place where their concerns can be voiced and debated, somewhere that's not all fizz and bang, but where there is discussion, thought and reflection."

Greenfield's views reflect a growing belief among Britain's science communicators of the need for a more 'bottom-up' approach. But the public understanding of science is just one initiative in what Greenfield promises to be a crowded agenda.

She is keen to find ways of supporting women returning to science after having children. She also plans to establish awards for science teachers, and lectures given by mid-career scientists who, although having good communication skills, would not under current practices be considered sufficiently well established to lecture at the Royal Institution.

It is an ambitious agenda, particularly as Greenfield plans to carry out her responsibilities at the Royal Institution for only part of the week. She is continuing with her neuroscience research at Oxford and with her involvement in Synaptica, a spin-off company developing drugs for Alzheimer's disease. She is also filming a BBC television series on the brain and the mind.

Ehsan Masood

UN climate organization faces criticism

[LONDON] The United Nations climate convention secretariat in Bonn has been heavily criticized for failing to follow standard UN procedures for managing its financial and administrative affairs.

In a report presented to the conference of the parties to the convention, which is being held this week in Buenos Aires, auditors from the UN Office of Internal Oversight Services found that staff were often untrained, and were overworked, particularly during the secretariat's move from Geneva to Bonn in August 1996.

In its reply, the secretariat says that most of the report's recommendations for addressing such problems have been implemented. But it notes that many recommendations cannot be fully implemented without extra administrative

staff, for which there is no provision in its budget.

Among the problems found by the auditors was the fact that the chief of administration during 1996 and 1997 had no experience in finance or accounting, and was not provided with a finance officer. They also found that proper selection procedures were not followed in recruiting consultants, and that their terms of reference were "sometimes very brief".

UN procedures state that the names of three candidates must be put forward for a consultant's post, with specific reasons for all recommendations. "In each of the cases we reviewed, only one candidate had been proposed for the contract, and no justification was provided for selection of the candidate," the report says.

E.M.

Breakthrough stirs US embryo debate...

[PARIS] One of the scientists behind last week's announcement of the culturing and differentiation of human embryonic stem cells says he is applying to the US National Institutes of Health (NIH) for funds to challenge the ban on federal funding for such research.

John Gearhart of the Johns Hopkins University School of Medicine in Baltimore, Maryland, will seek funding to study the differentiation of embryonic stem cells into blood and neuronal cells. The move will test whether the therapeutic promise of last week's breakthrough will weaken ideological and political resistance to human embryo research in the United States.

Many argue that the expanded scientific and therapeutic applications of human embryo research that will be opened up by the breakthrough will lead to a shift in public perception of the utility of such research, and pressure to relax the funding ban.

In an interview with *Nature*, Harold Varmus, the director of the NIH, said that embryonic stem cell research "is of sufficient magnitude and importance that the federal government should be playing an active role in supporting it".

The potential clinical applications opened up by the successful culturing of human embryonic stem cells — reported independently last week by Gearhart's team and by James Thomson's group at the University of Wisconsin — are broad. One



Gearhart: plans test case to challenge ban.

immediate application of the progenitor cells — which have the capacity to grow indefinitely and differentiate into all human cell types — would be to screen drugs on fully characterized human cell lines.

But ultimately, large quantities of disease-free cells might be produced for transplants, such as pancreatic beta cells for the treatment of diabetes, neuronal implants for Parkinson's disease, or cells for brain, nerve, and heart grafts (see *Nature* 391, 325; 1998).

The potential impact on fundamental developmental and cellular biology, including the generation of 'knock-out' cell lines, have also been unanimously acclaimed. "We are witnessing a coming of age of cell technology," says Ron McKay, head of the Laboratory of Molecular Biology at the US National Institute of Neurological Disorders and Stroke. "For me this is the future of molecular biology."

"It is probably the biggest development since recombinant DNA," says Jeremy Rifkin, president of the Washington-based Foundation on Economic Trends. He adds that the science is "highly valuable", although he is sceptical of the uses to which it will be put.

Previously, embryo research has been perceived as largely a means for improving *in vitro* fertilization procedures. But the prospect that it may underpin a broad generic technology for therapies in almost every disease area is likely to reignite the embryo research debate.

"Congressmen will not be able to tell sick and dying people that you can't do this or that because of moral reservations, say about surplus embryos," argues Arthur Kaplan, director of the Penn Center for Bioethics at the University of Pennsylvania.

At present, embryo research is banned from federal funding, where it could be openly regulated, but it is allowed to proceed largely unregulated within the private sector.

Varmus believes there will be a debate about the ban in Congress and in the public arena, and points out that congressional hearings are in the offing. Several researchers argue that the setback for Republicans in this month's congressional elections may embolden those who favour funding for embryo research. NIH lawyers are poring over the federal funding ban to determine whether cultures of embryonic stem cells might be interpreted as falling outside its scope.

In an editorial last weekend, *The New York Times* described the breakthrough as "not only a stunning achievement, but a rebuke to Congress for banning federal funding of this exciting research".

But in a statement to *Nature*, Congressman Jay Dickey (Republican, Arkansas), maintained his position that "the ban serves a very good purpose in our society because it honors the sanctity of life".

"There are no instances in which I feel the ban on federally funded research on human embryos should be lifted," says Dickey. "The language of this ban prevents taxpayer funding for bizarre experiments, such as cloning. Eventually, I could see the embryonic stem cell technology going in this direction."

But Gearhart is one of many researchers who complain about what they describe as an exclusive political and dogmatic emphasis on the morality of embryo research, and on its potential abuses. They say this is to the detriment of considered ethical discussion of the various forms of embryo research, the need to ensure that these are regulated appropriately, and the conditions required to exploit them fully for the public good.

In a bid to stir public debate, Gearhart says he intends to bring a test case to NIH. "The government has to move... to resolve this issue for everybody," he says.

Rifkin has launched a petition to Congress calling for a moratorium on all commercial exploitation of embryonic stem cells until the long term social and ethical consequences have been considered. **Declan Butler**

Company seeks strict controls on access

[PARIS] Excitement among biomedical researchers over the breakthrough in the culturing of human embryonic stem cells is giving way to concerns about the possible constraints that will be imposed on access to the technology.

The techniques developed by James Thomson's group at the University of Wisconsin have been patented by the Wisconsin Alumni Research Foundation, and licensed by Geron, a Californian biotechnology company. The company has licensed similar technology from the Johns Hopkins University School of Medicine, where John Gearhart's group works (see above).

Tom Okurma, Geron's

vice-president of research, says the company "intends to collaborate as widely as possible", but that cells would only be made available "under a very restrictive material transfer agreement". "We plan to selectively transfer the cells to folks that we know, that are in the field, and have a track record of appropriate ethical applications."

The agreement would not only protect the company's commercial interests, but also set ethical restrictions on the research that could be done — with a ban on human cloning, creation of chimaeras and modification of the germline, for example.

"The two big questions on my mind are, whether these cells are going to be widely available, and whether

people with NIH grants will be able to work on them," says Brigid Hogan, a cell biologist at Vanderbilt University School of Medicine in Nashville.

Arthur Kaplan, director of the Center for Bioethics at the University of Pennsylvania, says it is a "disaster" to have such fundamental technologies "privatized", arguing that this risks impeding publication and holding back development of the technology.

Jeremy Rifkin, president of the Foundation on Economic Trends, says he intends to challenge the Wisconsin patents at the US patent office. "There is no patent in history that is comparable in terms of the extraordinary monopoly conferred by this one," he complains. **D.B.**

...while Europe contemplates funding ban

[PARIS] As pressure grows for the United States to relax its ban on federal funding of human embryo research (see opposite), Europe is contemplating heading in the other direction and banning European Commission funding of research involving the destruction of human embryos.

The European Parliament is due to vote this month on an amendment that would ban the financing of such research within the European Union's Framework research programmes. A 'yes' vote would result in the issue going to negotiations between the parliament and the council of ministers, both of which must approve the Framework programme before it can be adopted.

Peter Liese, a German member of the parliament (European People's Party/Christian Democrat) and author of the amendment, argues that the union should only fund research whose utility and acceptability attracts a unanimous consensus among member states. "We should concentrate on research that we all agree is necessary," he says.

Where no consensus exists, the strictest legislation should apply, argues Liese, pointing out that human embryo research is banned in Germany, France and Austria. But this interpretation is contested by several members of the European Group on Ethics in Science and New Technologies. This body advises the commission on ethical matters, and is to release an opinion on the ethics of human embryo research this month.

The group is divided on the ethics of human embryo research. But Anne McLaren, of the Wellcome/CRC Institute in Cambridge, is one of several of the group's members who feel that a European-wide ruling is inappropriate, because member states have different attitudes to human embryo research, reflecting their cultural traditions.

"We would all be sorry to see funding of human embryo research prohibited within the fifth Framework programme," says McLaren. "In a multicultural assembly like Europe, it is not ethically acceptable for some countries to have a veto on funding that would apply to everyone else."

The commission's understanding is that it does not have the legal authority to impose a ban in cases where member states are divided, says one senior commission official. Few European Union research projects currently involve human embryo research, he says. But a ban would send a strong political message against such research.

This prospect has infuriated many researchers. "I'm so angry that I've written to the [UK] minister of health to protest," says Robert Edwards, the researcher responsible for Louisa Brown, the world's first test tube baby. "In my country I can do embryo research, and we passed bills in our parlia-



Pompidou: supports research if it is limited to using surplus embryos donated to science.

ment to allow this," he says, criticizing what he calls "an unholy alliance of German Lutherans, staunch Catholics and Green... trying to apply their rules across the board".

The outcome of the vote in the European Parliament is difficult to predict. A funding ban is likely to receive support from Roman Catholic and Green lobbies. But Alain Pompidou, a medical researcher and prominent

bioethicist who is a French member of the parliament, predicts that a blanket ban on funding of human embryo research is unlikely to pass unchallenged. A more likely prospect is the adoption of a diluted form of the amendment, he says.

Pompidou says he and many other parliamentarians would support allowing embryo research for therapeutic studies but limiting this to the use of surplus embryos that have been donated to science by couples. He would like the creation of embryos for research purposes to be outlawed.

The research opportunities opened up by the breakthroughs in the culture and differentiation of human embryonic stem cells announced last week have added further uncertainty to the debate. Liese describes these as "a new development that has to be examined". He is unsure whether such cells would fall under the proposed ban. In principle, cells could eventually be taken from an embryo without destroying it. **Declan Butler**

Swiss to vote on ban on *in vitro* fertilization

[ZURICH] Only months after Swiss voters rejected tough restrictions on research in genetic engineering, the controversial issue of *in vitro* fertilization (IVF) is scheduled to be put to a referendum.

Early next year, the Swiss people will be asked whether all assisted reproduction techniques, including IVF, should be banned. If they vote yes, the ban will be written into the constitution.

The referendum, tentatively scheduled for March 1999, was initiated by a group called the *Initiative für humane Fortpflanzung* (initiative for humane reproduction), which has gathered the 100,000 signatures needed to start referendum procedures.

Supporters of the referendum argue that IVF is being driven mainly by scientists who want to use spare IVF embryos for their research. Researchers dismiss this notion as fantasy, and opponents of the referendum argue that the initiative will lead infertile couples to seek help in other European countries, and so discriminates against those

who cannot afford to do so.

In an attempt to forestall public approval of a complete ban, the government has proposed strengthening the existing legislation on IVF procedures. New regulations would ban the donation of eggs and genetic screening of IVF embryos for inherited diseases before implantation.

The revisions to the law are expected to be approved by parliament early next month, and would be implemented if the referendum rejects an outright ban.

Some prominent Swiss researchers, however, while not opposed to some of the government's proposed revisions, are critical of the suggested ban on pre-implantation diagnosis. "Why would you let a woman go through this demanding procedure, only to see her get pregnant with a fatally or incurably sick child?" asks Walter Gehring, head of the department of cell biology at the Basel-based Biozentrum.

But the IVF working group of the Swiss Society for Fertility, Sterility and Family

Planning opposes all the government's proposed changes. Wolfgang Holzgreve, head of the Women's Clinic at Basel University Hospital, says the government's suggestion "shows that gut reactions towards medical sciences still dominate over rational decision-making". The working group, however, supports legislation forbidding research on human embryos.

Meanwhile, Swiss researchers have welcomed last week's announcement of the successful cultivation of human embryonic stem-cell lines (see opposite). But, given the proposed ban on research on humans, they are uncertain whether they will be able to use such cell lines for their own research.

The use of human embryonic stem-cell lines for research may already be illegal in some Swiss cantons, says Hermann Schmid, head of the project for fertility medicine at the Federal Department of Law and Policing. But he adds that a nationwide consensus is needed. **Ulrich Bahnson**

Europe's Mars mission wins approval – if funds can be found

[MUNICH] Mars Express, the European Space Agency's (ESA's) hastily planned mission to Mars, was last week given conditional approval by the agency's Science Programme Committee (SPC). But ESA may not be able to afford to fly the mission.

The SPC, which includes representatives of ESA's member states, agreed that Mars Express could only be built and flown if ESA's council agrees next month to maintain the purchasing power of the agency's science budget, which has been capped for the past three years. The council, however, is not expected to lift the cap.

One member of the SPC says that, although Mars Express is a scientifically sound mission, its financial requirements will not be allowed to delay other missions that have already been approved. These include the infrared mission FIRST and the microwave background mission Planck.

'Virtual university' project gets started in Spain

[BARCELONA] The Open University of Catalonia announced plans last week to launch a web-based project allowing home-

based students to take courses from universities around the world. No foreign-language skills will be necessary, as all material will be translated automatically into the student's language. A pilot programme of the so-called 'Metacampus' project will be launched next year.

At a second stage, the virtual campus network is expected to link up with university centres in Catalonia, Lombardia, Baden-Württemberg, Rhône-Alpes and Wales. According to the university's vice-chancellor, Gabriel Ferraté, a virtual 'world-wide' university system could be a achievable within five years. The main requirements are the political will to put it into practice, and agreement on topics such as registration processes, tuition fees and systems for validating credits.

Princeton physicist wins seat in Congress

[WASHINGTON] Rush Holt, a physicist and former assistant director of the Princeton Plasma Physics Laboratory in New Jersey, was elected last week to represent the Princeton area in the US Congress. Holt won the seat for the Democrats from Michael Pappas — one of only five sitting Republican members to lose their seats in the 3 November elections.

Holt has pledged to get Congress to address science-driven issues such as global warming and biodiversity conservation, and is expected to play an immediate role in its consideration of science-policy questions (see *Nature* 394, 411; 1998). Many had said that the son of a former US senator and the fourth scientist in the new Congress lacked sufficient money to fight the incumbent. But Holt was supported by individual contributions from many prominent US scientists as well as a late infusion of funds from the Democratic Party, and won by a 3 per cent margin.

Call for openness over hazardous technologies

[MUNICH] Decision-making on issues relating to potentially hazardous technologies, such as genetic engineering and nuclear technology, must become more democratic if major controversies about technical innovation are to be avoided. This is the conclusion of a study by the independent Stuttgart-based Centre of Technology Assessment, financed by the research ministry of Baden-Württemberg.

The study, which surveyed more than 1,500 people in Baden-Württemberg, found that most people are generally in favour of new technologies, but many fear that

industry could try to introduce high-risk applications without proper public consultation, and ignore public objections. More than a quarter of those surveyed said they would consider “unconventional” forms of protest against potential hazards, such as illegal demonstrations, occupation of buildings or sabotage.

Giacconi to take up US radioastronomy post

[WASHINGTON] After nearly six years as director-general of the European Southern Observatory, Riccardo Giacconi is planning to move to a new job. Next July he will take over as president of Associated Universities, Inc., a Washington-based non-profit corporation that since 1957 has operated the National Radio Astronomy Observatory for the National Science Foundation.

With headquarters in Charlottesville, Virginia, the observatory runs radiotelescopes at Green Bank, West Virginia, Socorro, New Mexico, and Tucson, Arizona, in addition to the Very Long Baseline Array of 10 telescopes across the United States.

A pioneer of X-ray astronomy, Giacconi was the first head of the Space Telescope Science Institute before moving to the European Southern Observatory.

US science groups seek yet more federal funds

[WASHINGTON] Budget posturing has started early this year in Washington. Less than a month after the 1999 appropriations were finalized, the heads of 33 scientific and engineering organizations have called on President Bill Clinton to present a budget for 2000 that “meets or exceeds” the goals of the Federal Research Investment Act, which passed the Senate last month but has yet to pass the House of Representatives.

The bill would double the federal investment in research and development over the next 12 years. Among the signatories of the 2 November letter to Clinton were the heads of the American Chemical Society, American Geophysical Union, American Institute of Physics, Association of American Universities, Federation of American Societies for Experimental Biology and National Association of Manufacturers.

Insurers win go-ahead for use of genetic data

[LONDON] The British government has bowed to the wishes of the insurance industry and rejected calls from the Human Genetics Advisory Commission for a moratorium on the use of genetic

information by life insurance companies.

The commission had sought a moratorium pending research into the implications of such use. But the industry argued that this was the first step towards a complete ban (see *Nature* 391, 3; 1998). Companies proposing to use information from a genetic test to work out life expectancy or the anticipated onset of ill health will now only need to have the test validated for accuracy by the government's Advisory Committee on Genetic Testing.

UK call to lift ban on cannabis in medicine

[LONDON] A British parliamentary committee called on the government this week to allow doctors to prescribe cannabis for medicinal use. It says this would boost research and help to remove the perceived stigma attached to the study of this field.

In a report published yesterday (11 November) the House of Lords Select Committee on Science and Technology says that although scientific evidence remains inconclusive, it has received enough anecdotal evidence “to convince us that cannabis almost certainly does have medical applications”. The committee says clinical trials should be set up for using cannabis to treat chronic pain and multiple sclerosis.

Coordinating European public health

Sir — I read with interest the account of the meeting of the international board of advisers for the proposed European Centre for Infectious Diseases (ECID), held in Montpellier, France, in September (*Nature* 395, 106; 1998). Declan Butler's article raises a relevant point, which is the potential clash between the creation of a Europe-wide public health agency (focused on infectious diseases) and the reluctance of each nation to cede sovereignty over public health policy.

This question was discussed at length at the meeting. Dan Colley, head of the parasitic diseases division of the US Centers for Disease Control (CDC) and a member of the ECID board of advisers, stressed that a parallel with the CDC can be made. In the United States, health policy is the

responsibility of each state. The CDC acts only as a coordinator, an adviser and a central data collector. The ECID could play a similar role in Europe, with health policy remaining under the sovereignty of each nation and the ECID providing complementary overall coordination.

The centre would build upon the many structures and initiatives that exist in Europe at national and international levels. However, it would create more efficient coordination at a global scale. This action should be extended to Eastern Europe and the former Soviet Union.

At the Montpellier meeting, a wall structure — where experts involved in research, surveillance and control of infectious diseases, and in training, would be concentrated in the same place — was

considered by most to be more attractive, synergistic and effective than a simple network. This view was supported by those representing developing countries, and it was agreed that the central structure should be complemented by outstations, either in Europe or in developing countries. It was also decided to extend the board of advisers to welcome epidemiologists and public health practitioners. A steering committee has been designated, with myself as coordinator. The board and committee will prepare a project plan within six months.

Michel Tibayrenc

Centre d'Etudes sur le Polymorphisme des

Micro-organismes,

UMR CNRS/ORSTOM 9926, ORSTOM, BP5045,

34032 Montpellier Cedex 1, France

e-mail: michel.tibayrenc@cepm.mpl.orstom.fr

Argentinian science down but not out

Sir — The letter from Marcelino Cerejido, an outstanding scientist, and my friend and former teacher, is misleading, damaging and insulting to Argentinian scientists (*Nature* 394, 314; 1998). No one would dispute the facts cited: scientists have been subject to that oppression and much more. But to adduce that all scientific activity in the country has to be dismissed is a dangerous *non sequitur*.

The economic difficulties engendered by privatization and the push for pseudo-cooperation with industry are diseases not exclusive to Argentina. That the present and past governments have had little interest in science and higher education is also not unique to this country.

Cerejido reaches an extraordinary conclusion: that Argentinians trained in this country, which is supposedly "devoid of science", achieve instant excellence simply by going abroad. Those who remain are doing "research, not science".

There are two possible corollaries. The first is that those who return to Argentina to work in science are so stupefied by society that they can no longer distinguish between good and bad science. However, this ignores the successes of many who are working hard with poor facilities. The second is that all scientists were in complicity with the former military regime. This ignores the difficulties of those who survived, and those who returned during the dark days of the regime. Their successes can be judged by the recognition accorded to them by eminent scientists and organizations

worldwide, as Cerejido admits.

Argentina is far from a paradise for science. Much more has to be done to generate more than a modest contribution in the present economic circumstances.

Cerejido imputes to Argentina an ideal that combines technology with theology. His fetish is misplaced.

J. Raul Grigera

(Director)

Instituto de Física de Líquidos y Sistemas Biológicos,

University of La Plata and CONICET,

c.c. 565, 1900 La Plata, Argentina

Time to take a healthier view of history

Sir — Ziauddin Sardar recalled Ottoman science, especially astronomy, under the title "Eclipsed no more"¹. On behalf of Turkish physicians, the progeny of Ottoman hakims, I would like to point out Ottoman medicine, which has also been largely neglected for the same reasons indicated by Sardar.

Although medical practice in the Turkish part of the Ottoman world was closely related to that of nearby countries such as Persia, especially from the ninth to the fifteenth century, it also developed its own distinct features. However, medical works were written mostly in Arabic and Persian. Classical texts were taught throughout the many hospitals and schools established by the Ottoman sultans, where patients were freely treated with traditional drugs, diet, hygienic and other supportive methods such as music for psychiatric patients. From the eleventh century

onwards, European medicine began to exert an influence in Turkish areas^{2,3}.

It is hoped that the project on "scientific literature in the Ottoman period"¹ will shed light on this neglected area.

Ibrahim C. Haznedaroglu

Hacettepe University Medical School, Ankara, Turkey

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No rustling at Roslin

Sir — Vandana Shiva has not only accused us in press reports and on her website of "stealing" and "patenting" the Indian Vechur breed of cattle but she says we are aiming at "total patriarchal control over reproduction" (*Nature* 394, 821; 1998)!

For the record, we at the Roslin Institute have never had any Vechur 'germplasm' (semen or embryos or even DNA), we have never worked on this breed or requested it, we have not applied for any patents relating to it, and none of our patents refers to Indian cattle breeds (this information is in the public domain and can be checked). I doubt anyway if a naturally occurring breed can be patented. We have never referred to the Vechur on our website and we have never received a letter from Moti Lal Madan of the Indian Council of Agricultural Research. We did, however, receive a request for information from Dr Sulochana, dean of Kerala Agricultural University (KAU), and I faxed him my full denial. Sosamma Iype of KAU has never been to Roslin, nor do we collaborate with KAU.

Grahame Bulfield

Roslin Institute, Roslin, Midlothian EH25 9PS, UK

Rickettsia, typhus and the mitochondrial connection

Michael W. Gray

The genome sequence of *Rickettsia prowazekii*, the agent that causes typhus, has been determined. What emerges is a snapshot of genome re-tailoring in a parasitic bacterium, and a new look at the evolutionary connection between *Rickettsia* and mitochondria.

On page 133 of this issue, Andersson *et al.*¹ report the complete genome sequence (1,111,523 base pairs) of *Rickettsia prowazekii*, the causative agent of louse-borne typhus. This bacterium and its relatives represent one of biology's great ironies. On the one hand, the historical ancestors of *R. prowazekii* precipitated some of the greatest plagues to afflict the human race (see box overleaf). On the other hand, an evolutionary antecedent of *R. prowazekii* participated in one of the seminal events in the evolution of eukaryotic (nucleus-containing) cells — the formation of mitochondria², cellular organelles that contain their own DNA and, during oxidative breakdown of glucose, produce the ATP that powers these cells. With the complete genome sequence of *R. prowazekii*, we can now examine this

important genetic blueprint for clues both as to what makes *R. prowazekii* such a great killer, and what allowed one of its ancestors to contribute so fundamentally to the emergence of eukaryotic cells in the first place.

R. prowazekii is an obligate intracellular parasite — that is, it can only live within other cells. Its gene content, like that of other parasitic eubacteria, has been reduced and tailored to suit its dependent lifestyle. Andersson *et al.*¹ have found that the *R. prowazekii* genome encodes 834 complete open reading frames, DNA sequences that specify protein sequences. This number is far less than the 4,288 protein-coding genes found in the fourfold larger genome of *Escherichia coli*, its free-living cousin³. However, *R. prowazekii* contains ten times as many genes as the most bacteria-like

mitochondrial genome described to date, the 69,034-bp mitochondrial (mt)DNA of the freshwater protozoon *Reclinomonas americana*⁴. Surprisingly, the *R. prowazekii* genome also contains the highest fraction of non-coding DNA (24%) found in any microbial genome so far, much of which may represent inactive genes that have been degraded by mutation, but have not yet been eliminated from the genome.

By comparing the sequences of bacterial and mitochondrial genes, we get the best evidence that *Rickettsia* and mitochondria are specific evolutionary relatives. Evolutionary trees based on small-subunit ribosomal RNA (SSU rRNA) originally pinpointed members of the α -division of the so-called 'purple bacteria' (proteobacteria) as the closest contemporary bacterial relatives of mitochondria⁵. More recent SSU rRNA trees divide the α -proteobacteria into two groups, with the rickettsial sub-division (to which *R. prowazekii* belongs) the one that is specifically affiliated with mitochondria² (Fig. 1).

The same result is seen with evolutionary trees based on mitochondrial proteins encoded by the nuclear genome⁶. Such nuclear genes are assumed to have been transferred from mitochondria during the drastic down-sizing that characterized evolution of the mitochondrial genome after it was acquired by the eukaryotic cell². In their analysis, Andersson *et al.*¹ constructed evolutionary trees comparing the amino-acid sequences encoded by mitochondrial and bacterial genes involved in energy metabolism (subunits of NADH dehydrogenase) and genetic processes (ribosomal proteins). True to expectation, these results show that *R. prowazekii* is more closely related to mitochondria than is any other bacterium whose genome has been investigated at this level of detail.

Andersson and colleagues rightly point out that the DNAs of *Rickettsia* and mitochondria are "stunning examples of highly derived genomes, the products of several modes of reductive evolution". Both lack genes for metabolizing sugars in the absence of oxygen (anaerobic glycolysis), as well as all or most of the genes involved in synthesizing amino acids and nucleotides. However, the *R. prowazekii* genome contains a complete set of genes encoding components of the tricarboxylic acid cycle, a metabolic pathway involved in respiration, and respiratory-chain complexes. A subset of the same genes is found in mtDNA, with the remainder in the nuclear genome. The functional profiles of *Rickettsia* and mitochondria are strikingly similar, with production of ATP occurring in basically the same way in the two systems.

Do these similarities mean that mitochondria evolved directly from a *Rickettsia*-like organism that was already highly reduced? The answer is almost certainly no. If one compares the organization of the same

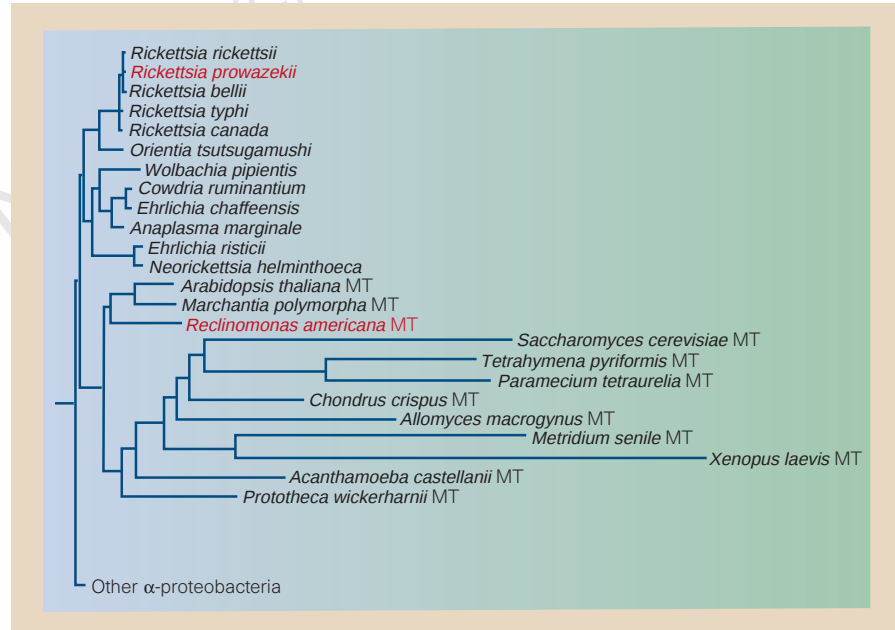


Figure 1 Relationship between the *R. prowazekii* genome and mitochondrial DNA. The tree shown is the α -proteobacterial/mitochondrial (MT) portion of a eubacterial/organellar small-subunit (SSU) ribosomal RNA tree. Extreme differences in the rate of mitochondrial sequence divergence are responsible for the separation of mitochondria into 'short-branch' (plants, *Reclinomonas americana*) and 'long-branch' groups. (The analysis used an aligned data set of 275 published eubacterial and organellar SSU rRNA sequences, and the tree was generated using DNADIST from Phylip version 3.5 (ref. 9) with the ML option and default parameters². Courtesy of D. F. Spencer, Dalhousie University.)

Rickettsia in medicine and history¹⁰⁻¹²

The Rickettsiae are obligate bacterial parasites – they multiply only within the cells of susceptible animals. They are responsible for a variety of insect-borne human diseases characterized by acute onset, fever, delirium and skin rash. *Rickettsia prowazekii*, the causative agent of louse-borne epidemic typhus, is named after H. T. Ricketts and S. J. M. Prowazek, both of whom died of typhus while studying rickettsial diseases early this century. Charles

Nicolle fared rather better, receiving the Nobel prize in 1928 for his discovery that epidemic typhus is transmitted by lice.

Although the plague of Athens in 430 BC was probably a typhus epidemic, an accurate medical description of the disease did not appear until the mid-1600s. The word typhus (from the Greek *typhos*, meaning smoky or hazy, and used by Hippocrates to describe a "confused state of intellect with a tendency to stupor") was not applied

to typhus fever itself until 1760; moreover, typhus was not clearly distinguished from typhoid fever until the mid-1800s.

Typhus ranks as one of the main epidemic diseases of human history, a truly apocalyptic pestilence that follows in the wake of wars, famine and other human misfortune. It has been estimated that between 1918 and 1922, the typhus epidemics in eastern Europe and Russia resulted in 20–30 million cases and at least 3 million

deaths. Medical personnel have been prominent as victims of this disease: in the 1915 Serbian epidemic, nearly all of that country's 400 doctors contracted typhus and more than a quarter of them died. Often, typhus determined the outcome of military campaigns more effectively than the actual battles themselves. In 1741, for example, Prague was surrendered to the French army because 30,000 of the opposing Austrians died of typhus.

M.W.G.

genes in the *R. prowazekii*¹, *E. coli*² and *Reclinomonas mitochondrial*⁴ genomes, vestiges of bacterial operon organization (gene clustering) are still clearly seen in the *Reclinomonas* mtDNA. However, the *Reclinomonas* mitochondrial and *R. prowazekii* genomes do not specifically share any derived characteristics of gene organization; indeed, certain gene clusters are uniquely rearranged in the *Rickettsia* genome, relative to what was probably the ancestral bacterial order. These comparisons emphasize that the *Rickettsia* and mitochondrial genomes independently descended from an α -proteobacteria-like ancestor, each undergoing a separate process of reductive evolution. The observed congruence in the functional profiles of *Rickettsia* and mitochondria is certainly intriguing, but it remains to be seen whether this seeming example of convergent evolution is more than a coincidence.

By examining other rickettsial genomes we should learn more about genome reduction in bacterial parasites, a challenging question in its own right. However, such studies are unlikely to provide more information about the nature of the genome in the most recent common ancestor of mitochondria and the Rickettsiae. While the search continues for organisms with mtDNAs that are even more ancestral than in *Reclinomonas*, it will be important to identify and explore the genomes of those minimally diverged, free-living α -proteobacteria that are specific but more distant relatives of both the Rickettsiae and mitochondria. Such genomes should yield additional clues relevant to the origin and evolution of mitochondria, a process that is central to the emergence of eukaryotic life^{7,8}. □

Michael W. Gray is at the Program in Evolutionary Biology, Canadian Institute for Advanced Research, Department of Biochemistry, Dalhousie University, Halifax, Nova Scotia B3H 4H7, Canada.
e-mail: M.W.Gray@Dal.Ca

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Superconductors

Mesoscopic magnetism

Manfred Sigrist

On page 144 of this issue¹, Geim and colleagues present measurements made on superconducting aluminium discs as small as 0.3 μm across. They show that some superconductors have a peculiar attraction for magnetic fields.

When metals become superconducting, not only does their resistance drop to zero, but their magnetic properties also change markedly: magnetic fields are expelled from the material's interior. Often this phenomenon is more readily measurable and more revealing about the nature of superconductivity than a material's electrical properties. Magnetic fields may still penetrate into so-called type-II superconductors, in vortices of circulating supercurrent (the resistance-free current carried by paired electrons). Nevertheless, the magnetic field is always diminished inside a superconductor relative to the external field — that is, superconductors are diamagnetic².

So it came as a surprise when highly disordered granular $\text{Bi}_2\text{Sr}_2\text{CaCu}_2\text{O}_8$ — one of the most prominent high-temperature superconductors — was found to have a paramagnetic response to a small external field^{3,4}. In this case the applied magnetic field is not expelled: on the contrary it is increased in the superconducting state. Several proposals have

been made for the origin of this unusual behaviour, called the paramagnetic Meissner effect (PME) or the Wohleben effect.

It seems fairly certain that the PME is a property of the granular structure of these superconductors with grains of 'mesoscopic' size — their scale is in between that of the microscopic crystal structure and that of bulk samples. Some theories of the PME are based on the unconventional symmetry of the superconducting state in high-temperature superconductors.

To test this idea, the IBM research group of Kirtley, Tsuei and co-workers made miniature loops (about 50 μm across) of various high-temperature superconductors⁵ (Fig. 1). These were designed to 'frustrate' the phase of the quantum-mechanical wavefunction that describes the superconducting electron pairs — that is, to prevent the phase from remaining constant, usually the most stable situation. This only works in superconductors with an unconventional electron-pairing symmetry (so-called *d*-wave pairing, in contrast to the *s*-wave pairing of standard superconductors such as niobium, lead and aluminium).

Kirtley *et al.* found that the frustration led to spontaneous supercurrents running without dissipation around the loop. These

generate a magnetic flux amounting to half the standard flux quantum $hc/2e (= 2 \times 10^{-7} \text{ G cm}^{-2})$ of a superconductor, equivalent to a magnetic moment exceeding that of the electron by many orders of magnitude. Strangely, this device does not have a stable state with zero magnetic flux. The magnetic moment of such a superconducting loop should indeed act like a paramagnet, as it aligns parallel to the external field in a similar manner to a magnetic needle. So this device is an example of a system displaying the PME.

The effect in this case is a quantum coherence phenomenon specific to the superconducting state of high-temperature superconductors. No effect of this kind has been observed in any conventional superconductor. The observation of this frustration phenomenon was crucial in proving the unconventional symmetry of the superconducting state in high-temperature superconductors — the chief motivation for performing this experiment⁶.

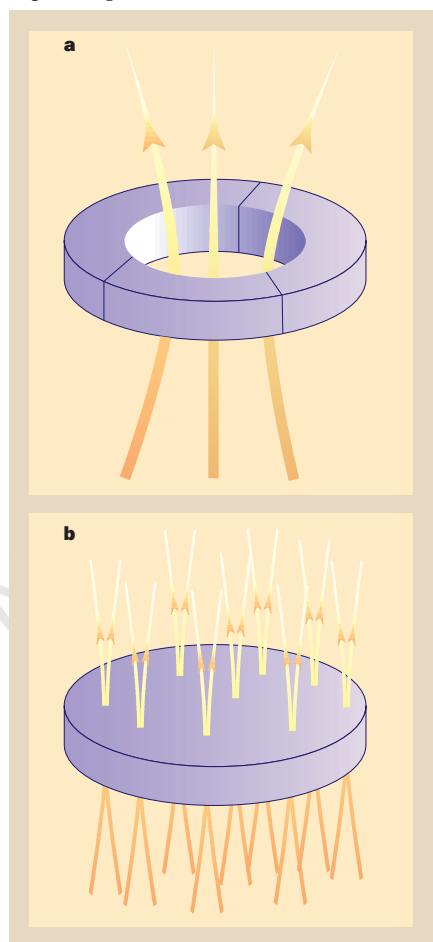


Figure 1 Magnetism in mesoscopic superconductors. **a**, A loop made of three crystals of a high-temperature superconductor⁵ frustrates the phase of the superconducting state. As a result a spontaneous current flows generating a magnetic flux. **b**, A mesoscopic superconducting aluminium disc¹ in an external magnetic field hosts various vortex patterns, including some metastable magnetic states. Both mechanisms can lead to paramagnetism.

But during the past few years, several groups have also reported paramagnetic responses in millimetre-sized single crystals of conventional superconductors such as niobium. Again, to test ideas about how this could happen in mesoscopic grains, we need a highly controlled experiment on these small scales. Geim and co-workers¹ have now demonstrated paramagnetism in micrometre-scale discs of aluminium, a substance that is superconducting below 1.2 K.

The miniaturization means that very few magnetic vortices are present, and there is a discrete set of states consisting of different arrangements of vortices which may be characterized by 'quantum' numbers, similar to those describing electron states in atoms. Most of these states are metastable, but may become stable if a magnetic field of the right strength is applied externally.

Geim *et al.* triggered transitions between different vortex states in their aluminium disc by varying the magnetic field. That produces large changes in the magnetization of the superconductor and so in the number of magnetic vortices threaded through the superconducting disc. Here, the PME originates from one of the metastable states with a large number of vortices, which must decay upon application of a strong external disturbance.

This property of metastability distinguishes the PME in the current-carrying loop and the disc, as the former is clearly in a stable state, like a magnetic needle in a magnetic field. The PME of granular high-temperature superconductors corresponds to the stable rather than the metastable situation, and appears to be caused by large magnetic

moments like those generated by the frustrated current loops⁷. Presumably the loops here consist of several connected grains. And the presence of spontaneous magnetic moments in zero external field has been directly observed⁸ in granular $\text{Bi}_2\text{Sr}_2\text{CaCu}_2\text{O}_8$ samples which show the PME.

But the metastable vortex states seen in the superconducting discs might also contribute to the PME of the granular high-temperature superconductors. Indeed, the effect is increased in samples where the grain structure favours states with densely-packed vortices. However, the same samples also provide good conditions for frustrated loop formation.

Further experiments on mesoscopic discs will teach us about vortex physics in conventional superconductors, and may enable us to observe some of the unusual vortex structures expected in unconventional superconductors. These miniature superconductors may also become important technological materials, largely because of their magnetic properties, and new devices may be designed to exploit these quantum coherence phenomena. □

Manfred Sgrist is at the Yukawa Institute for Theoretical Physics, Kyoto University, Kyoto 606-1, Japan.

e-mail: sgrist@yukawa.kyoto-u.ac.jp

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Protein transport

Life and death of a signal peptide

Gunnar von Heijne

Signal peptides target proteins for secretion in both prokaryotic and eukaryotic cells. A deceptively simple-looking amino-terminal extension on the newly synthesized polypeptide chain, the signal peptide takes part in an array of protein–protein and protein–lipid interactions. The result is initiation of protein translocation through a proteinaceous channel — the translocon — in the bacterial inner membrane, or in the endoplasmic reticulum (ER) of eukaryotic cells. After fulfilling its mission, the signal peptide receives its *coup de grâce* from the signal peptidase, a membrane-bound enzyme that liberates the mature protein from this now useless appendage.

Thanks to the work of Paetzel *et al.*¹, who report the crystal structure of a bacterial signal peptidase on page 186 of this issue, we now have a glimpse of how this unusual

enzyme can cleave the signal peptide so precisely as it emerges from the relative safety of the membrane. Not only do we see a new protease fold and a strikingly hydrophobic surface patch that positions the active site relative to the lipid bilayer, but the structure of the bacterial enzyme can also be used as a template to model other signal peptidases, and will be of great interest for designing new antibiotics.

Signal peptides have a common structure: a short, positively charged amino-terminal region (n-region); a central hydrophobic region (h-region); and a more polar carboxy-terminal region (c-region) containing the site that is cleaved by the signal peptidase (Fig. 1, overleaf). It has been obvious all along that many of the interactions between the signal peptide and other molecules must be hydrophobic in nature, but only recently has the structural basis for at least

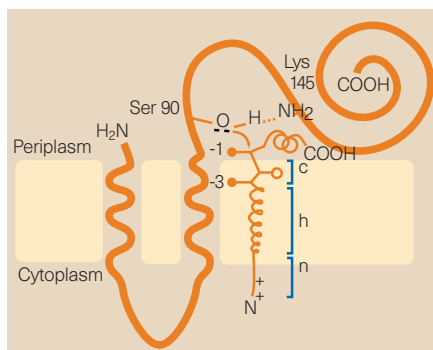


Figure 1 The signal peptidase of *Escherichia coli*, the structure of which has been solved by Paetzel *et al.*¹. Two amino-terminal transmembrane helices anchor the protein in the inner membrane, and the catalytic carboxy-terminal domain is partly immersed in the outer leaflet of the bilayer. Serine-90 (Ser 90) is the acylating nucleophile and lysine-145 (Lys 145) acts as the general base in both the acylation and deacylation steps of catalysis. Small residues in positions -1 and -3 in the signal peptide bind to the S1 and S3 specificity pockets in the enzyme. The h-region is helical, whereas the c-region must be in an extended conformation to be accessible for cleavage.

some of these interactions become apparent.

As the signal peptide emerges from the ribosome, it is first recognized by a ribonucleoprotein complex, the signal recognition particle (SRP). The subunit that binds the signal peptide has a hydrophobic surface groove lined by flexible methionine side chains, and it seems perfectly designed to adapt to the highly variable h-region². By virtue of its affinity for the membrane-bound SRP receptor, the SRP ensures that the nascent protein is delivered to the translocon. Here, the signal peptide is scrutinized a second time, and it is eventually inserted in a lipid-exposed location between two transmembrane helices of the SecYEG protein^{3,4}. In the ER translocon, this insertion step correlates with the establishment of a tight seal between the ribosome and the translocation channel⁵. As seen by electron microscopy⁶, the nascent chain runs in a closed, continuous tunnel from the ribosomal P-site through the large ribosomal subunit and then through the translocation channel, finally emerging in the lumen of the ER. In *Escherichia coli*, where proteins are translocated after they have dissociated from the ribosome, the SecA protein seals the channel from the cytoplasmic side and helps move the nascent chain through the translocon⁷. An X-ray structure of the SecA protein has been shown at meetings, but so far has not been published.

At this point, the signal peptide spans the membrane with its carboxy-terminal end facing the ER lumen (or, in *E. coli*, the periplasm). Although still associated with the translocon, it is also exposed to mem-

brane lipids and its h-region has a helical conformation³. Enter the signal peptidase. Its job: to skim the luminal (or periplasmic) surface of the membrane, looking for suitably exposed signal-peptide cleavage sites. Paetzel and colleagues' structure of the periplasmic domain of the *E. coli* enzyme¹ tells us how this is accomplished.

First, an extended hydrophobic patch surrounds the active site, suggesting that this part of the enzyme is immersed in the outer leaflet of the lipid bilayer. Second, the structure of the active site readily explains the requirement for small residues such as alanine in positions -1 and -3 , upstream of the cleavage site, and also shows that the c-region must be in an extended conformation. The h-region thus, presumably, positions the c-region near the lipid head-groups, within reach of the signal peptidase. This may also explain why signal peptidase does not cleave transmembrane helices in integral membrane proteins, or signal peptides with artificially lengthened h-regions⁸. Such helices generally extend across the lipid head-group region, so they do not present the required extended conformation.

This is where the story could end — the last mopping up is taken care of by various oligopeptidases, which digest the signal peptide into free amino acids. But biology would not be so interesting if it didn't always come up with the unexpected. Certain signal peptides leak back into the cell where they bind to

proteins such as calmodulin⁹, or they are presented to the immune system by molecules of the major histocompatibility complex on the surface of the cell¹⁰. So, some signal peptides probably have a second signalling function, distinct from their role in targeting.

We are experiencing a new wave of structural and biochemical work on protein targeting, which is finally showing us the intimate details of the life and death of signal peptides and the machineries that they put in motion. The key is the h-region — a simple stretch of about ten hydrophobic residues that primes the SRP, unlocks the translocon and positions the signal peptide for cleavage. The messenger is the message. □

Gunnar von Heijne is in the Department of Biochemistry, Arrhenius Laboratories, Stockholm University, S-106 91 Stockholm, Sweden.

e-mail: gunnar@biokemi.su.se

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Chronology

Friday XIII

This is the Friday the Thirteenth Club, meeting in Paris in 1930 to dance underneath a ladder and carry open umbrellas indoors. Being a rational reader of *Nature*, you surely applaud this contemptuous attitude towards superstition, so you won't be at all concerned by the following sinister tale.

In 1582, Pope Gregory XIII introduced a new calendar to replace the old Julian system, whose inaccuracies had made Easter slip slowly through the seasons. To bring the festivals back to their old positions, ten days disappeared from October 1582. Some people thought the days were being stolen from them.

But rebuilding and resetting the calendar had a more subtle effect. The Gregorian cycle of 400 years contains exactly 20,871 weeks, and hidden in the calendar's machinery is a bias towards certain days of the week landing on certain dates in the month. The 13th is more likely to be a Friday than any other day (Brown, B. H. *Amer. Math. Monthly* **40**, 607; 1933).

Bernard Yallop now points out that with a personal computer it is possible to look for such peculiarities "without



resorting to mathematics" (*Spectrum* October, 66; 1998). His table shows for example that there are 688 Friday-the-thirteenths every 400 years, but only 684 Thursdays; and a month (like a week) is most likely to begin on a Sunday.

Did the Friday the Thirteenth Club know of their good fortune in having these extra opportunities to carouse? I only hope they didn't meet a sticky end before finding out.

Stephen Battersby

**100 YEARS AGO**

An interesting pamphlet upon the temperance question, from the pen of Dr Archdall Reid, has just reached us. ... According to Dr Reid the longer a race has had alcohol, and the easier and more abundant the supply, the more sober it is. For instance, the grape-growing southern Europeans are at the present time more sober than the races of northern Europe, where alcohol is more difficult to obtain, although formerly they were quite as drunken. ... According to Dr Reid this diminution of the craving for alcohol has been produced by the action of natural selection working in the presence of an abundant supply of the harmful substance in question. Any cause which reduces the supply of alcohol, or in any way increases the difficulty of obtaining it, in that it hampers the action of natural selection, tends to perpetuate drunkenness ... This truly dreadful picture of the world, or rather all races not yet immune becoming "thoroughly drunken before they can hope to become thoroughly sober," can, to some extent, be mitigated by artificial selection. The innate drunkard, when found out by letting everybody have free access to alcohol, must be treated as a lunatic, and above all not be allowed to procreate. By this means the alcohol tainted "germ plasm" will finally be eliminated, and the race will become immune to alcohol.

From *Nature* 10 November 1898.

50 YEARS AGO

A dytiscid beetle which fell about three feet off the laboratory bench to the floor of the old malaria Bureau, Kuala Lumpur, displayed a unique type of bioluminescence. The beetle appeared to emit bright flashes of white light, three or four at a time, from its eyes. The fluorescent type of bioluminescence is invisible even in weak light; but these flashes were conspicuous in competition with the light from a fairly bright electric bulb on the laboratory bench. ... Mr Williamson tentatively suggests that the flashes from the beetle's eyes were caused by the reversal of the normal process of vision, energy in the form of light having been emitted instead of being absorbed by retinal pigment, as the result of intermittent outwardly directed nervous impulses due to shock.

From *Nature* 13 November 1948.

Solar physics**The Sun at small scales**

John H. Thomas

The Sun is a unique laboratory for studying particular astrophysical processes, such as the interaction of thermal convection with a magnetic field. But many important phenomena in the solar atmosphere are thought to occur on length scales smaller than the resolution limit of current solar telescopes (about 0.2 arcseconds, or 140 km). Hence the quest for higher resolution, and hence a workshop* on the topic.

The energy generated by nuclear fusion in the Sun's core is transported outward first by radiation, and then through the outermost layers by thermal convection. The Sun's magnetic field, generated by a dynamo mechanism at the base of the convective zone, is transported to the surface by diffusion and buoyancy. Near the surface the turbulent convection and the magnetic field interact as a complicated nonlinear system. This interaction produces fine-scale structuring of the magnetic field, which in turn dominates nearly all aspects of solar activity, including coronal heating, flares and mass ejections, and radio, X-ray and γ -ray emission.

In some respects theory now leads observations in high-resolution solar physics. For example, numerical simulations of the interaction of granular convection with a magnetic field show the formation of intense magnetic flux tubes in the intergranular lanes on scales smaller than can currently be resolved (O. Steiner, Kiepenheuer Inst. Solar Physics). These tubes account for much of the magnetic flux at the solar surface, and are crucial to our understanding of solar magnetism and activity.

Numerical simulations of solar granulation, pioneered by Nordlund¹, show the formation of localized, supersonic downdrafts in the dark intergranular lanes. These downdrafts produce strong shocks and acoustic noise just below the solar surface, and are thought to be the main source of excitation of the global acoustic oscillations^{2,3} which provide the basis for helioseismology. The rapid initiation of these convective downdrafts by localized radiative cooling, accompanied by an inflow from the surroundings, may actually dominate the excitation process (M. Rast, High Altitude Observ.). Observations have disclosed such localized acoustic events on the Sun, and show that they have sufficient power to drive the global oscillations^{4,5}. Comparisons between observed spectral-line profiles of these events, and synthetic profiles generated from numerical

*High-Resolution Solar Physics, 19th National Solar Observatory/Sacramento Peak Summer Workshop, Sunspot, New Mexico, 28 September–2 October, 1998.

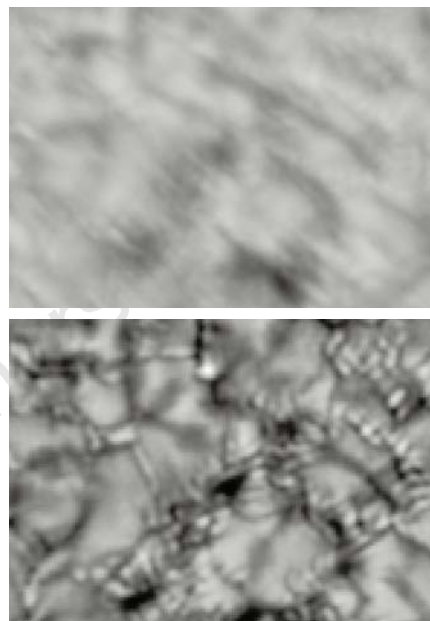


Figure 1 Uncorrected (top) and corrected images of solar granulation using a technique that combines phase-diversity and speckle-imaging concepts. The image size is 12.7 by 9.3 arcseconds, and the corrected image has a spatial resolution of less than 0.2 arcseconds, nearly at the 0.18-arcsecond diffraction limit of the 76-cm aperture Dunn Solar Telescope at NSO.

simulations of granular convection, are in good agreement (P. Goode, New Jersey Inst. Technol.).

The best place to take high-resolution observations of the Sun is, of course, from space. But new techniques for correcting the distortion of wavefronts caused by fluctuations in the Earth's atmosphere offer improved 'seeing' from ground-based telescopes. This correction is more difficult for solar than for stellar images because of the lack of a point source as a reference to measure the wavefront distortions. Nevertheless, progress is being made using images of the solar granulation pattern as a reference. The ultimate goal is to correct solar images in real time using wavefront sensors and adaptive optics. As described at the meeting, adaptive optics systems are under development at the Dunn Solar Telescope at the National Solar Observatory (NSO)/Sacramento Peak (T. Rimmele), and also at the Swedish Vacuum Solar Telescope on La Palma (G. Scharmer), which is expected to be replaced soon by a new telescope with double the aperture (95 cm).

In the meantime, various *post facto* methods of image reconstruction are delivering impressive results. Speckle interferometry combined with narrow-band polarimetry

has yielded resolved magnetograms of the small, intense magnetic elements in the quiet solar network (the boundaries of supergranular convection cells), with diameters of 0.2 arcseconds (ref. 6); enough of these flux tubes have now been observed to show statistically that at least half of them have diameters smaller than that (C. Keller, NSO).

The method of phase diversity, in which simultaneous pairs of in-focus and out-of-focus images are used to estimate and correct wavefront distortions, has also proved effective. A technique that combines speckle and phase-diversity methods (R. Paxman, J. Seldin, ERIM Int., Ann Arbor, MI; C. Keller, NSO) has produced sharp, nearly diffraction-limited images of the solar granulation (Fig. 1). This method uses a rapid time-sequence of phase-diversity image pairs to obtain a maximum-likelihood estimate of the true, undistorted image. The corrected images fully resolve many of the small bright points in the dark intergranular lanes, which are known to correspond to intense magnetic flux tubes. The brightest point in the corrected image in Fig. 1 has an intensity of a little over twice the average photospheric intensity.

Above the solar photosphere, in the chromosphere and corona, the structuring of the atmosphere is expected to be on larger scales. Surprisingly, however, NASA's Transition Region and Coronal Explorer (TRACE) mission has now revealed a wealth of fine structure in the corona. The TRACE satellite, launched in April 1998, has returned more than 700,000 images of the Sun at various wavelengths, and Fig. 2 shows a beautiful array of coronal loops with unprecedented resolution (A. Title, Lockheed-Martin Solar and Astrophys. Lab.). Most of the loops have transverse dimensions of about an arcsecond (the resolution limit of the TRACE telescope), regardless of their length. Movies made from long time-sequences of such images show them to be in constant motion, dancing in response to shaking of their footpoints by the five-minute acoustic oscillations at the photosphere.

Also seen in Fig. 2 is a coronal emission

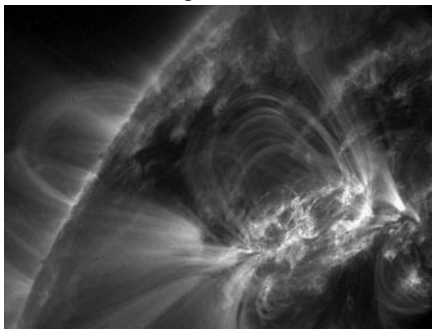


Figure 2 Ultraviolet image (in a narrow wavelength band around the Fe IX 171-Å line) of the solar corona from the TRACE satellite. The thin, bright coronal loops, which follow magnetic field lines, are generally only about one arcsecond wide, and are in constant motion.

feature first resolved by the TRACE mission, a low-lying, cellular network of bright plasma dubbed the 'moss'. This structure is about 2,000 km thick and is heated to coronal temperatures (10^6 K) in spite of being only some 2,000–3,000 km above the solar surface. Comparison of TRACE images with ground-based observations taken at the Swedish Vacuum Solar Telescope show that the moss is generally associated with bright regions ('plages') in the underlying photosphere, and that the dark component of the cellular moss pattern is associated with fibril-like upward jets of cool gas, the well-known 'spicules' (T. Berger, Lockheed-Martin). TRACE images also show transient, bright, cusp-shaped features that have been tentatively identified as dissipative magnetic reconnection events which heat the corona.

A further push on the limits of high resolution will come from a new-generation, ground-based solar telescope with an aperture of 3 m or more, planned by the NSO (J.

Beckers, C. Keller, NSO). A large aperture is required not only for improved angular resolution, but also just to collect enough photons. The high spectral and temporal resolution needed to study small-scale solar phenomena, together with the multiple images and short exposures required for adaptive optics or image reconstruction, can easily leave solar observers starved for photons — a situation that will surely amuse the night-time observer of distant stars. □

John H. Thomas is in the Departments of Physics and Astronomy, and Mechanical Engineering, University of Rochester, Rochester, New York 14627, USA.

e-mail: thomas@astro.me.rochester.edu

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Developmental genetics

Too much sex is bad for males

Amanda Swain and Robin Lovell-Badge

Many species rely on chromosome-based systems — such as one X versus two X chromosomes — to trigger the differences between males and females. To do this they must find ways to count the sex chromosomes and to activate the process of dosage compensation, which corrects the imbalance in sex-linked genes. A paper by Carmi, Koczynski and Meyer¹ on page 168 of this issue brings us closer to understanding chromosome counting in the nematode worm *Caenorhabditis elegans*. The authors have identified an X-linked gene that encodes a protein related to nuclear-hormone receptors. This protein, SEX-1, represses the transcription of *xol-1*, the pivotal gene involved in both dosage compensation and sex determination.

The XOL-1 protein is a repressor that is downregulated to allow XX embryos to develop as hermaphrodites, and to dosage compensate by reducing the transcription of genes on both X chromosomes. Conversely, XOL-1 is upregulated in XO embryos to a level ten times as high as in XX embryos². This triggers male development and leaves X-expression at its maximum rate. Studying the regulation of *xol-1* is a way to understand how the number of X chromosomes is perceived *in vivo*.

Previous studies identified three regions of the X chromosome thought to contain important counting elements. When these regions are deleted, almost all XO embryos die owing to defects in dosage compensation. A gene that maps to one of these regions, *fox-1*, has been shown² to be

involved in post-transcriptional regulation of *xol-1*. However, mutations in *fox-1* alone have very weak effects on sex determination and dosage compensation, and it is clear that transcriptional mechanisms are largely responsible for the different levels of XOL-1 activity between males and females.

Carmi *et al.*¹ devised a genetic screen to find regulators of *xol-1*, and identified two X-linked and 16 autosomal mutations. One of the X-linked mutations was a previously identified gene of unknown function, originally termed CNR14 but now renamed *sex-1*. Using worms with either point mutations in *sex-1* or a duplication in the region of the X chromosome to which *sex-1* maps, the authors found that extra copies of *sex-1* tended to be lethal to XO but not to XX embryos, whereas a lack of *sex-1* was lethal to XX but not XO embryos. (Embryos that did not die had phenotypes characteristic of those seen with mutations in other dosage-compensation or sex-determination genes.) Reassuringly, the phenotype caused by the lack of *sex-1* in XX embryos could be suppressed by *xol-1* null mutations, indicating that *xol-1* is the only critical target of SEX-1.

This all fits with the importance of *sex-1* as a counting element. However, the phenotypes were not particularly strong and lethal, it was never 100%. One might predict that the loss of one *sex-1* allele in XX embryos should lead to sex reversal and no dosage compensation, as these embryos would be equivalent to XO embryos as regards the dose of SEX-1. But this was not the case, and the authors needed to remove both copies of

sex-1 to see an effect in XX embryos. This could be used to argue that *sex-1* is not a critical gene in counting. Yet when *fox-1* function (which, by itself, has a very weak effect) is also removed from *sex-1* homozygous XX embryos, they all die. Therefore, *sex-1* is clearly important in the counting mechanism, although it is obviously not sufficient and requires *fox-1* plus at least one other gene. This makes the story confusing, but not surprising for those of us who work with mice and expect redundancy! Perhaps the other X-linked gene identified by the screen is another missing counting element, with a direct effect on *xol-1*.

The genetics argues that SEX-1 represses transcription of *xol-1*, but does it act directly? To answer this question, Carmi *et al.* devised an ingenious strategy involving transgenic worms, which allowed them to visualize the location of multicopy arrays of the *xol-1* promoter within the nucleus. Using an antibody to SEX-1 they showed that the endogenous protein specifically co-localized with these promoter arrays. These results indicate that SEX-1 associates with chromatin that contains *xol-1* DNA, consistent with a direct regulatory interaction.

The authors show that SEX-1 is mainly required zygotically, as predicted, to repress only the sex-specific transcription of *xol-1*, which occurs maximally during gastrulation in the early embryo. But how does SEX-1 act as a repressor? Some nuclear-hormone receptors repress transcription in a ligand-dependent manner³, but SEX-1 is unlikely to act in this way because its ligand-binding domain is predicted to fold differently from those of known ligand-dependent nuclear-hormone receptors. Perhaps it folds in such a way that it constitutively recruits co-repressors, thereby silencing transcription. Indeed, Rev-Erb, which is closely related to SEX-1, lacks the ligand-dependent transcriptional-activation domain AF-2, and acts as a repressor — possibly by competing for DNA-binding sites and inhibiting activation by other nuclear-hormone receptors⁴. Some repressors show differences in the DNA-binding domain and may act by forming heterodimers with other, activating nuclear-hormone receptors, again preventing them from binding DNA. Alternatively, they may bind to these other nuclear-hormone receptors and recruit co-repressors to the site of transcription. It is interesting to speculate that *C. elegans* may have another activating nuclear-hormone receptor, present at the same time as SEX-1, that activates *xol-1* transcription and is antagonized by SEX-1.

But how does the *xol-1* promoter distinguish between one or two copies of X-chromosome counting elements such as SEX-1? Some transcription factors can work as either activators or repressors in a concentration-dependent manner. For example, the zinc-finger-type transcription factor Kruppel,

which is important for patterning of the early *Drosophila* embryo, is present in a concentration gradient. It represses target sequences at high concentrations, when it forms homodimers, yet activates those same sequences at lower concentrations when it is present as monomers⁵. However, this may be a special case and it is, perhaps, easier to imagine that SEX-1 competes against other transcription factors that work as activators. The autosomal mutations identified in the original screen could correspond to gain-of-function mutations in such genes, although it is more likely that these are recessive mutations in co-factors required for SEX-1 function.

Carmi *et al.*¹ comment on an intriguing parallel with mammalian sex determination, where a nuclear-hormone receptor, DAX1, represses male development in a dose-dependent manner⁶. The *Dax1* gene is also X-linked, but there is no evidence that it is involved in any X-chromosome counting mechanism (which is not linked to sex determination in eutherian mammals). In fact, *Dax1* is autosomal in marsupials, where some sexual characteristics — such as the choice between making a pouch or a scrotum — are determined by X-chromosome dosage⁷. DAX1 seems to work against the action of SF1, which is an activating nuclear-hormone receptor. However, it also behaves antagonistically to SRY, the product of the Y-linked testis-determining gene⁶. Presumably SRY and SF1 act in a complex, to promote the expression of critical secondary testis determinants, and high levels of DAX1 can interfere with this. So, to understand how SEX-1 functions in *C. elegans*, it is now especially important to define the other transcription factors that act on the *xol-1* promoter.

There is one other intriguing connection between SEX-1 and DAX1. Followers of *Star Trek* and its offshoot *Deep Space 9* will know that one of the characters is Commander Jadzia Dax, who represents a species called a Trill. She comprises the host body of a young woman, Jadzia, and a 300-year-old symbiont life form named Dax, which happens to be a worm. Obviously *C. elegans* with a compatible sex-determining mechanism. □

Amanda Swain is in the Section of Gene Function and Regulation, Chester Beatty Laboratories, Institute of Cancer Research, 237 Fulham Road, London SW3 6JB, UK.

e-mail: aswain@icr.ac.uk

Robin Lovell-Badge is in the Division of Developmental Genetics, MRC National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK.

e-mail: rlovell@nimr.mrc.ac.uk

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Daedalus

Glass is for ever

Modern information-storage systems are highly wonderful. But the most capacious of them (disks and tapes) still depend on mechanical scanning. Daedalus is dreaming up a read-only high-volume storage system with no moving parts.

His starting point is fibre optics. A mere gram of glass, in the form of fibre 1 micrometre in diameter, would extend about 500 km. Lay a binary code along it at 1 bit/micrometre, and it would store 500 gigabits — an unprecedented solid-state information density. You could write on the fibre as it was being drawn, by passing it through an ion beam modulated with the information to be stored. The ions would diffuse into the hot surface, and would be fixed when the fibre cooled to room temperature. Cunningly, Daedalus wants these ionic impurities to be fluorescent. Illuminated by light of one frequency, they will re-radiate it at a second frequency.

To read the fibre, Daedalus will simply send a short intense optical pulse down it. As the pulse passes each 'bit' of data, it will generate a fluorescent pulse, which will travel back along the fibre to a detection unit at the pulse-injector. The returning pattern of fluorescence pulses contains all the information in the fibre.

In this simple design, the loss of energy to fluorescence would rapidly damp out the most intense interrogation-pulse. But fibres these days can have optical gain. An erbium-doped fibre acts as a distributed laser; illuminated with a suitable pump radiation, it actively amplifies the pulses travelling along it. Indeed, the data-points in the new fibre might even be encoded as spots of erbium doping.

Daedalus's Fibre Read-Only Memory, or FROM, will hold its information unalterably and permanently, as a pattern of impurity doping along its length. Simple, fast, capacious and secure, FROMs will form the basis of libraries and archives of all kinds. Unlike disks or tapes, they cannot deteriorate or go wrong, nor become unplayable by format changes to their reading mechanism. FROMs will be entrusted with the most fundamental, unchanging information: historical and public records, encyclopaedias, runs of scientific journals, great works of art. Authors and artists will no longer be satisfied with mere paper publication or world-wide broadcast distribution. The ultimate cachet of worth will be to have one's creation immortalized in the only truly time-proof form — a FROM.

David Jones

Obituary

Demetrios Papahadjopoulos (1934–98)

Innovation in liposomes

Demetrios Papahadjopoulos, who died in San Francisco on 21 September, was a pioneer in the development of liposomes and their applications. One of these is their use as a system for the efficient, targeted delivery of drugs and vaccines, leading to optimal pharmacological action.

Papahadjopoulos was born on 24 August 1934 in Patra, which has been one of Greece's western gates for more than 2,500 years. He obtained a BSc in chemistry from the University of Athens and a PhD in biochemistry from the University of Washington in Seattle, under D. J. Hanahan. His interest in phospholipids and their role in blood coagulation (the subject of his thesis) brought him in 1966 to A. D. Bangham's laboratory at the Institute of Animal Physiology, Babraham, Cambridgeshire, where liposomes had just been discovered. His fascination with them was to remain undiminished throughout his life.

Liposomes had originally been recognized as closed, semipermeable bilayers of phospholipids (very similar to cell membranes) that can entrap water and solutes. Some of Papahadjopoulos's work, both in Babraham, and later on at the Roswell Park Memorial Institute in Buffalo, New York state, dealt with the development of novel, reproducible methods for making liposomes of a defined vesicle-size distribution. He was thus able to study bilayer permeability to ions, which was found to reach a maximum at the liquid crystalline transition temperature of the phospholipid. This research greatly contributed to the understanding of the physical nature of liposomes (and, indirectly, of cell membrane biophysics) in terms of their structure, the stability of the bilayer and mobility of the lipids within it, their interaction with proteins, and membrane fusion. Liposome research had made the transition from art to science, and Papahadjopoulos's contribution to it was decisive.

In the early 1970s, liposome research branched into biology and therapeutics when it was shown that liposomes could be a way to deliver entrapped drugs and vaccines to cell targets *in vivo*, thus avoiding many of the problems associated with direct drug use. At Roswell Park Memorial and, from 1978, at the Cancer Research Institute of the University of California at San Francisco, Papahadjopoulos and his group were quick



to appreciate the significance of this new angle. Among the central questions they addressed was that of how liposomes are taken up by cells. Their elucidation of the mechanisms concerned, by observing the migration of gold-containing vesicles from the coated pits on the cell's membrane to the cell organelles known as lysosomes, was particularly influential. These studies were much facilitated by the development in the Papahadjopoulos laboratory of a method for the efficient entrapment of solutes in liposomes, the so-called reverse-phase evaporation technique. This allowed the encapsulation of substantial amounts of material in the liposomes' aqueous phase.

Soon after the study of liposome uptake by cells, Papahadjopoulos started investigating the possibility of using liposomes for DNA transfer. In 1981, two papers appeared (one from Papahadjopoulos's laboratory) reporting on their use for the transfer of DNA into cultured cells. Initially, however, transfection efficiency was low, and much work was done in his laboratory to improve efficiency by manipulating the lipid composition of liposomes and the conditions under which they were incubated. In 1983, the first reports of liposome-mediated gene transfer *in vivo*, and of successful expression of those genes, began to appear independently. This line of research has culminated in a potentially effective clinical approach to gene therapy and genetic immunization.

Another aspect of the drug-carrier concept of liposomes that captured the imagination of many a liposomologist was the prospect of using them for the ligand-mediated targeting of drugs to selected cells. The approach consists of attaching to the liposome surface molecules, or fragments of molecules, that have a particular affinity for other, specific molecules on the target-cell surface. This too was an area where Papahadjopoulos excelled, with several of his techniques on the conjugation of cell-specific antibodies to the liposomal surface being widely adopted.

The capacity of liposomes to deliver relatively large amounts of drug, with the help of only a few molecules of ligands, is an advantage over the use of ligands conjugated to the drugs themselves. But there were several drawbacks to the approach, mainly related to the immune reaction prompted by the ligand and further potentiated by the liposomal carrier. Moreover, targeted, as well as conventional, liposomes are relatively rapidly cleared from the blood circulation and intercepted by cells of the reticuloendothelial system (mostly fixed macrophages) before they can reach their intended targets.

Papahadjopoulos and co-workers tackled the latter problem, and to some extent circumvented it, first by the use of gangliosides added to the liposomal surface; and, later (independently with several other groups), with polyethyleneglycol in the same role. His group dominated this second approach and was able to produce liposome formulations that persisted in the circulation long enough to deliver therapeutic doses of drugs to tumours. The approach led eventually to the liposome-based drug Doxil, licensed for clinical use and marketed by Sequus Pharmaceuticals (of which Papahadjopoulos was a co-founder).

Demetrios Papahadjopoulos — Demetri, to his friends and colleagues — was justifiably proud of the many young people he trained, not only in his chosen branch of science, but also in scientific integrity, the need for precision and reproducibility in experimentation and, above all, in what Pasteur defined as the origin of scientific creativity: *Savoir s'étonner à propos* (to know when to be astonished). His was a multifaceted personality, for he was also artistically sensitive (and part-owner of an art gallery), and with a high degree of historical, political and philosophical awareness. For those qualities, and his intellect and warmth, Demetri will be sorely missed — by his many friends and colleagues, and by his wife Brigitte Sternberg-Papahadjopoulos, and three sons from a previous marriage.

Claude Nicolau and Gregory Gregoriadis

Claude Nicolau is at Harvard Medical School, 1256 Soldiers Field Road, Boston, Massachusetts 02125, USA, and the Louis Pasteur University, Strasbourg.

e-mail: cnicolau@aol.com

Gregory Gregoriadis is at the Centre for Drug Delivery Research, The School of Pharmacy, University of London, 29–39 Brunswick Square, London WC1N 1AX, UK.

e-mail: Gregoriadis@cua.ulsop.ac.uk

Cell suicide for beginners

Martin Raff

The ability to commit suicide is a fundamental property of animal cells. This overview considers recent progress in understanding the nature of the suicide process and how it is controlled.

Cell death is an invariable part of animal development, and it often continues into adulthood. In a mature human, for example, millions of cells die every minute; we remain the same size only because, by an unknown mechanism, cell division exactly balances cell death. It seems peculiarly wasteful for so many cells to die, especially as most of them are perfectly healthy at the time of their demise. What purpose does this carnage serve?

In some cases the answer is clear¹. During development, cell death helps sculpt parts of the body, carving out cavities or separating digits, for example; it also eliminates structures that once served a function, but are no longer needed, such as the tail of the tadpole when the tadpole becomes a frog. Throughout vertebrate life, cell death eliminates most newly formed lymphocytes (the principal cells of the immune system), including those that are useless or potentially dangerous. It also helps to control cell numbers by removing excess cells: neutrophils (a type of white blood cell), for instance, are produced continuously in the bone marrow, but the vast majority die there within a few days without ever functioning². This apparently futile cycle of production and destruction presumably serves to maintain a ready supply of cells that can be mobilized to fight infection wherever it occurs in the body. Compared with the life of the organism, cells are apparently cheap.

Based on the characteristic way cells look when they die in different circumstances, it was proposed in 1972 that normal cell deaths, as well as some pathological ones, are suicides³. That is, the cells activate an intracellular death programme and kill themselves in a controlled way — a process now known as programmed cell death, or apoptosis. Apoptotic cells shrink and are rapidly eaten by neighbouring cells, before there is any leakage of their contents. Because they are eaten and digested so quickly, there are usually few dead cells to be seen, even when large numbers of cells have died. This is probably why apoptosis was neglected for so long and why its extent is almost certainly still underestimated. By contrast, cells that die accidentally in response to an acute injury often do so by an uncontrolled process called cell necrosis: they swell and burst,

spilling their contents over their neighbours and eliciting a damaging inflammatory response.

The idea that animal cells have a built-in death programme was a crucial insight, but its general acceptance took another 20 years. That came only when studies of the nematode worm *Caenorhabditis elegans* identified genes dedicated to apoptosis and its control¹; then, with the discovery of similar genes with similar functions in humans^{4,5}, the cell death field was radically transformed — from neglect to hysteria in one or two years.

Mechanism

The studies in *C. elegans* identified two genes, *ced-3* and *ced-4* (*ced* for cell death abnormal), required for apoptosis in the worm: if either gene is inactivated by mutation, the 131 cell deaths that normally happen during the development of the worm (which has only about 1,000 cells when mature) fail to occur¹. Remarkably, the mutant worms with 131 extra cells have a normal life span, showing that in this organism apoptosis is not essential for either life or normal ageing. By contrast, more complex animals cannot survive without apoptosis: mutations that inhibit apoptosis in the fruit fly *Drosophila melanogaster*, for example, are lethal early in development⁶, as are mutations in mice that inhibit apoptosis mainly in

the developing brain⁷.

The protein encoded by the *ced-3* gene was found to be very similar to a human protein called interleukin-1-converting enzyme (ICE)⁴. ICE is an intracellular protein-cleaving enzyme (a protease) that cuts out interleukin-1, a signalling protein that induces inflammation, from a larger precursor protein⁸. The similarity between the CED-3 and ICE proteins was the first indication that the death programme depends on protein cleavage (proteolysis).

Soon new members of the CED-3/ICE family of proteases were identified, including more than ten in humans, many of which become activated in apoptosis⁸. They all have the amino acid cysteine in their active site and cleave their target proteins at specific aspartic acids, and so are called caspases⁹. Each is made as a large, inactive precursor (a procaspase), which is itself activated by cleavage at aspartic acids, usually by another caspase⁸ (Fig. 1). In apoptosis, caspases are thought to be activated in an amplifying proteolytic cascade, cleaving one another in sequence.

Once activated, some of the caspases cleave other specific proteins in the cell to help kill the cell quickly and neatly⁸. They cleave proteins supporting the nuclear membrane, for example, thereby helping to dismantle the nucleus; they cleave a protein that normally holds a DNA-degrading enzyme (a DNase) in an inactive form, freeing the DNase to cut up the DNA in the cell's nucleus¹⁰; they cleave protein constituents of the cell's skeleton and other proteins involved in the attachment of cells to their neighbours, thereby helping the dying cell to detach and round up, making it easy to ingest; and so on.

One of the earliest changes in apoptosis is the movement of a negatively charged phospholipid molecule (phosphatidylserine) from the inner to the outer surface of

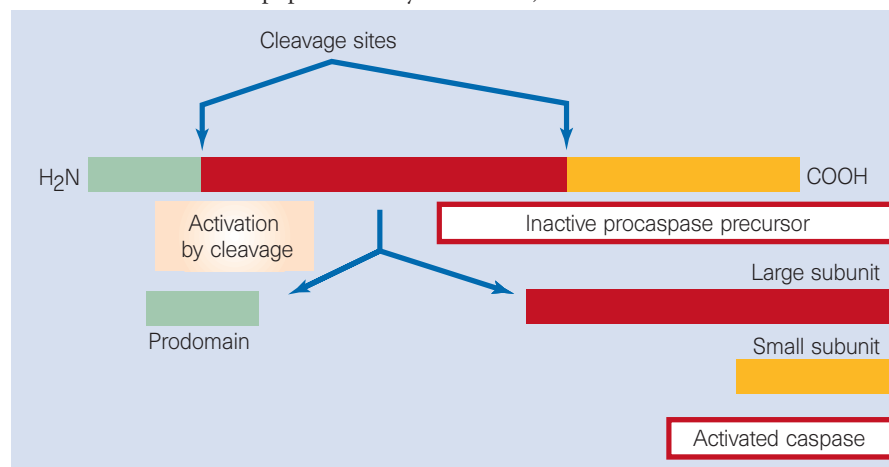


Figure 1 Activation of a caspase. The protein is made as a large inactive precursor (a procaspase), which is activated by cleavage at two aspartic acids. The 'prodomain' is discarded, and the large and small subunits form the active enzyme. It is thought that there are two large and two small subunits in an activated caspase (not shown). The activating cleavages are usually catalysed by caspase or procaspase molecules themselves.

the cell membrane, where it helps mark the cell surface so that the dying cell is quickly recognized and eaten by a neighbouring cell¹¹. It is still uncertain how caspase activation leads to this lipid rearrangement.

The caspases required for apoptosis vary depending on the cell type. When the mouse gene encoding caspase-3 is inactivated, for example, normal apoptosis fails to occur in some cells in the developing brain. In consequence, the mouse dies around birth with a deformed brain that contains too many cells; apoptosis occurs normally, however, in most of the other organs of such mice⁷. The caspases required for apoptosis in the same cell type can also vary depending on the stimulus that induces the process: caspase-9, for instance, is required for some lymphocytes to undergo apoptosis in response to gamma irradiation but not to ultraviolet irradiation¹². Some caspases such as ICE (caspase-1) seem not to be directly involved in apoptosis at all, but instead help generate inflammatory signals such as interleukin-1⁸.

Activation

The procaspases and other proteins required to carry out the death programme are made continuously by healthy cells, from the earliest stages of an animal's development. Thus the suicide machinery is always in place; all that is needed is a trigger to activate it¹³. How, then, is the death programme activated? There are only a few cases where the mechanism is known.

When we become infected by a virus, some lymphocytes become activated and induce the infected cells to kill themselves (thereby preventing the virus from multiplying and spreading to other cells). The lymphocytes secrete various proteins onto the surface of the infected cell: one of them (perforin) assembles into transmembrane channels that allow other proteins to enter the cell; one of these (granzyme B) is a protease that cleaves and activates certain procaspases to begin the proteolytic death cascade¹⁴ (Fig. 2a).

Killer lymphocytes have a second way of inducing cells to kill themselves. They produce a protein (Fas ligand) that binds to receptors (Fas) on the surface of the target cell, causing the receptors to aggregate¹⁵. The aggregated receptors recruit adaptor proteins from the cytoplasm, which, in turn, recruit procaspase-8 molecules into the aggregate; the clustered procaspase molecules cleave and activate each other to begin the suicide sequence¹⁶ (Fig. 2b). This killing mechanism is used, for example, to eliminate normal activated lymphocytes after they have done their job, which helps to terminate immune responses¹⁵. Remarkably, stressed cells sometimes insert both Fas and Fas ligand into their surface membrane, and use them to activate procaspases and kill themselves.

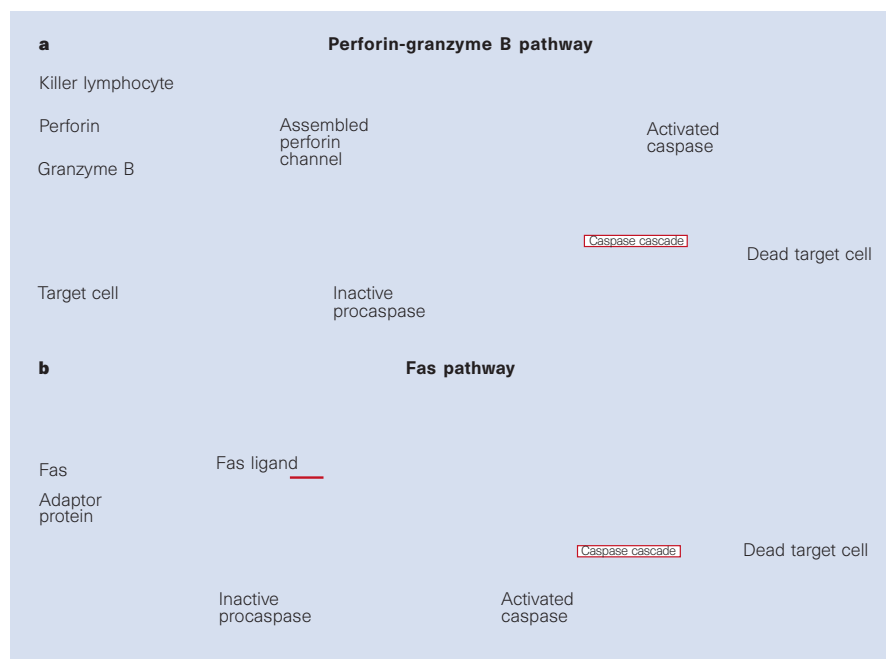


Figure 2 Two ways that a lymphocyte can kill a target cell. In both cases procaspases are activated in the target cell, leading to apoptosis. a, The lymphocyte secretes proteins onto the target cell surface: the perforin protein assembles into channels that enable the granzyme B protein to enter the target cell and activate procaspases. b, Fas ligand on the lymphocyte surface binds to the Fas protein on the target cell, causing the Fas molecules to form clusters. An adaptor protein then binds to the clustered Fas molecules and recruits inactive procaspase-8 molecules, which then activate one another to initiate the caspase cascade.

Procaspases can also become activated without a stimulating signal at the cell surface, especially if a cell is stressed or damaged. A cell can somehow recognize when one of its parts is damaged and, if the damage is severe enough, it will activate procaspases from within the cell and kill itself. In one case, the mechanism concerned has been uncovered. When mitochondria are damaged, say by a toxic drug, they can release the protein cytochrome *c*. This protein normally functions in the electron-transport processes in mitochondria that generate most of the cell's ATP, the main energy carrier in cells. Once it is in the cytoplasm, however, cytochrome *c* has an entirely different function. It binds to an adaptor molecule similar to the CED-4 protein required for apoptosis in *C. elegans*. The formation of this complex leads directly to the activation of procaspase-9, which initiates a caspase cascade, leading to

apoptosis¹⁷ (Fig. 3).

Surprisingly, cytochrome *c* also escapes from mitochondria and activates procaspase-9 in this way in many other cases of apoptosis, whether the apoptosis is triggered from outside or inside the cell¹⁸. Although the mechanism of escape is uncertain, the released cytochrome *c* amplifies the caspase cascade, increasing the speed and efficiency of the death process.

Procaspases can also become activated when other parts of the cell are stressed or damaged, but the mechanisms involved are less well understood. Damaged DNA in the nucleus, for example, can activate apoptosis, as can the accumulation of misfolded proteins in the endoplasmic reticulum, where many lipids and proteins are made. One mechanism that triggers apoptosis when DNA is damaged depends on an intensely studied cancer-suppressing protein called

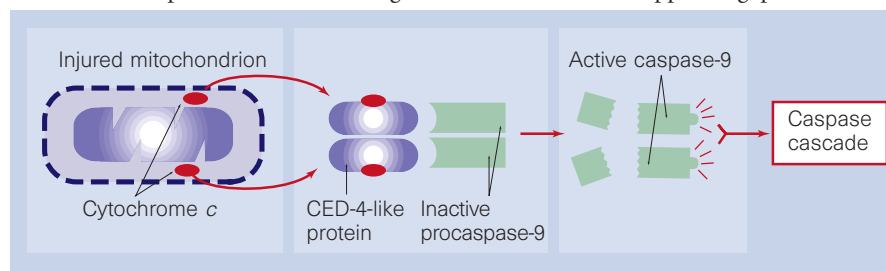


Figure 3 How the release of cytochrome *c* from mitochondria contributes to apoptosis. The released cytochrome *c* molecules bind to CED-4-like adaptor proteins. The adaptor proteins then aggregate and bind procaspase-9 molecules. The clustered procaspase-9 molecules activate one another, and the activated caspase-9 molecules then activate other procaspases, leading to apoptosis.

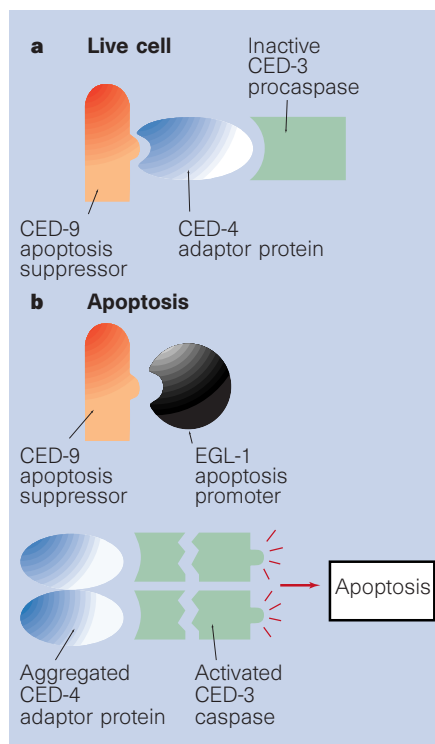


Figure 4 How Bcl-2 family proteins are thought to determine whether a cell lives or dies in *C. elegans*. **a**, In cells that live, the CED-9 protein inhibits the CED-4 protein from aggregating and activating the CED-3 procaspase. **b**, In cells that die, an apoptosis-promoter of the Bcl-2 family (EGL-1) binds to CED-9, thereby releasing the complex of CED-4 and CED-3. The CED-4 can now aggregate, which brings the bound CED-3 procaspase molecules close enough together so that they can activate each other. The complexes are probably bound to intracellular membranes by CED-9.

p53, which accumulates and becomes activated in response to DNA damage; by various routes, the p53 can either stop cell division or induce apoptosis¹⁹. The gene encoding p53 is inactivated by mutation in over 50% of human cancers, which allows the cancer cells to survive and proliferate even when their DNA is damaged; in this way the cancer cells accumulate more mutations that increase their malignancy.

Intracellular controls

A decision to die should not be taken lightly, and so it is not surprising that the death programme is regulated in complex ways, both from inside and outside the cell. A major class of intracellular regulators is the Bcl-2 family of proteins, which, like the caspase family, has been conserved in evolution from worms to humans²⁰. The *ced-9* gene in *C. elegans* encodes such a protein: if it is inactivated, most of the cells in the developing worm die, and the worm therefore dies early in development; but, if *ced-3* or *ced-4* are also inactivated so that apoptosis cannot occur, the worm and all of its cells live⁵. So it seems that the

only reason any cells in the developing worm live is that *ced-9* normally keeps the death programme suppressed in these cells.

The *ced-9* gene is similar to the human *bcl-2* gene, which was initially identified in a common form of lymphocyte cancer, where a chromosome abnormality causes excessive production of the Bcl-2 protein. The high levels of Bcl-2 promote cancer by inhibiting apoptosis, thereby prolonging cell survival. Remarkably, the human *bcl-2* gene is so similar to *ced-9* that it can suppress apoptosis in *C. elegans* when artificially introduced into the worm²¹.

Fifteen Bcl-2 family members have been identified so far in mammals. Some, such as Bcl-2 and Bcl-X, suppress apoptosis; others, such as Bax, Bad and Bid, promote it²⁰. Some of these proteins can bind to each other; when an apoptosis-suppressor forms a complex with an apoptosis-promoter, each protein inhibits the other's function. The ratio of suppressors to promoters helps determine a cell's susceptibility to apoptosis²².

The Bcl-2 family proteins regulate apoptosis in several ways^{20,22}. Some act simply by binding to and inhibiting apoptosis-suppressors: in *C. elegans*, for example, such a protein is required to block the inhibitory effects of the CED-9 protein and allow apoptosis to occur in the 131 cells that normally die²³ (Fig. 4). Other family members, however, act in more sophisticated ways. CED-9, for example, acts as an apoptosis-suppressor in worms in two ways: it binds directly to the CED-3 caspase, blocking its activity, and it binds to CED-4, blocking its ability to activate the CED-3 procaspase²⁴ (Fig. 4).

None of the known mammalian Bcl-2 family apoptosis-suppressors binds directly to caspases, however, although they can inhibit caspase activation indirectly²⁰. Some bind to the CED-4-like adaptor protein and block its ability to activate procaspase-9; some inhibit the release of cytochrome *c* from mitochondria; and some bind to the apoptosis-promoters and inhibit their function.

Many proteins of the Bcl-2 family have a hydrophobic tail that enables them to bind to the outside surface of mitochondria, the endoplasmic reticulum and the nucleus, and they are thought to function mainly on these organelles. Bax, for example, acts as an apoptosis-promoter, in part at least, by binding to mitochondria, where it can interfere with mitochondrial function and release cytochrome *c*¹⁸.

The activity of Bcl-2 family members can be regulated by an increase or decrease in the expression of the gene that encodes them or by modification of the proteins themselves, by either the addition of phosphate (phosphorylation) or cleavage by proteases. The cleavage of Bcl-2 by caspases, for instance, inactivates Bcl-2's inhibitory function, while the cleavage of the apoptosis-promoter Bid by caspases

releases a fragment that triggers the release of cytochrome *c* from mitochondria; both events accelerate the death process^{25,26}.

Another important family of intracellular apoptosis regulators are the IAP (inhibitors of apoptosis) proteins. Some of these bind to caspases or procaspases and inhibit them directly. If one of the two known IAP genes in *Drosophila* is inactivated by mutation, all of the cells in the fly embryo undergo apoptosis (H. Steller, personal communication).

Extracellular controls

Like most functions of animal cells, the death programme is regulated by signals from other cells, which can either activate it or suppress it. These cell-cell interactions are part of the complex 'social' controls that ensure that individual cells behave for the good of the animal as a whole — in this case, by surviving when they are needed and by killing themselves when they are not. For the vast majority of normal cells that undergo apoptosis, however, we know little about the signals that tilt the balance towards death. But there are exceptions.

A classic example is the apoptosis that causes the tadpole's tail to disappear at metamorphosis, which is triggered by a surge of thyroid hormone in the bloodstream. In other cases, certain developing cells produce signals that cause only neighbouring cells to die; this seems to be the way that cells are eliminated between developing fingers and toes. The challenge in the latter cases is to understand how these localized apoptosis-inducing signals are produced at precisely the right place and time.

Other normal cell deaths occur because the cells fail to receive enough apoptosis-suppressing signals (also called survival factors) from other cells. Most of our cells seem to require such signals: if a cell is experimentally isolated from its neighbours, it will undergo apoptosis unless it is supplied with the appropriate survival factors. Apparently, the only thing our cells can do on their own is kill themselves, and the only reason they normally remain alive is that other cells are constantly stimulating them to live. This surprising arrangement is thought to ensure that cells normally only survive when and where they are needed²⁷.

Dependence on survival factors also provides a simple strategy for regulating cell numbers. During vertebrate development, for example, more nerve cells are produced than are needed, and they compete for the limited amounts of survival factors produced by the target cells that they normally connect with. Only some of the nerve cells get enough factor to survive; the rest undergo apoptosis, thereby automatically matching their numbers to the number of target cells²⁸.

How do extracellular survival factors,

which generally bind to cell-surface receptor proteins, suppress apoptosis? In some cases they activate the production of an apoptosis-suppressor such as Bcl-2 or Bcl-X. In others they block an apoptosis-promoter such as Bad, by activating enzymes (protein kinases) that phosphorylate the protein: unphosphorylated Bad binds and inhibits the apoptosis-suppressing proteins Bcl-2 and Bcl-X, but, when phosphorylated, it releases these proteins so that they can suppress apoptosis²⁹ (Fig. 5). In most cases, however, the mechanism is unknown.

Cell suicide in non-animal cells

Programmed cell death also occurs in plants — during development, in the senescence of flowers and leaves, and in the response to injury and infection. It is still uncertain whether caspases are involved in any of these cell deaths. Even some unicellular organisms can die in a way that looks like apoptosis, but the molecular mechanisms are not known.

The best understood example of non-animal cell suicide occurs in bacteria. Some strains of *Escherichia coli* produce an inactive form of a protease. If they become infected by a particular virus, one of the viral proteins binds to the protease and activates it. The activated protease cleaves and inactivates a bacterial protein required for protein synthesis, shutting down synthesis and killing the bacterium — thereby curtailing viral multiplication and protecting nearby *E. coli* from infection³⁰. Although this bacterial death programme shares a number of features with caspase-dependent apoptosis in animal cells, the proteases involved in the two programmes are unrelated. Other cell suicide programmes in unicellular organisms, however, may have provided the starting point for the evolution of the caspase-dependent programme in animal cells.

Cell death in disease

There are many disorders where cells die prematurely: heart cells die in a heart attack, for example, and brain cells in a stroke. In these acute conditions, many cells die by necrosis. But some of the less badly damaged ones die by apoptosis, and the search is on for drugs that block the process in the hope that some of the apoptotic cell deaths can be prevented.

Many viruses have evolved strategies for blocking apoptosis, so as to prevent infected cells from committing suicide before the virus has had a chance to multiply. Some viruses produce proteins that bind to caspases and block their activity. Although these can be used experimentally to inhibit caspases, they are too large to enter cells, and one has either to inject them into the cell or to introduce the genes that encode them. A more convenient strategy is to synthesize modified small peptides that can enter cells and block the active site of caspases⁸. Such inhibitors are now widely used by cell biolo-

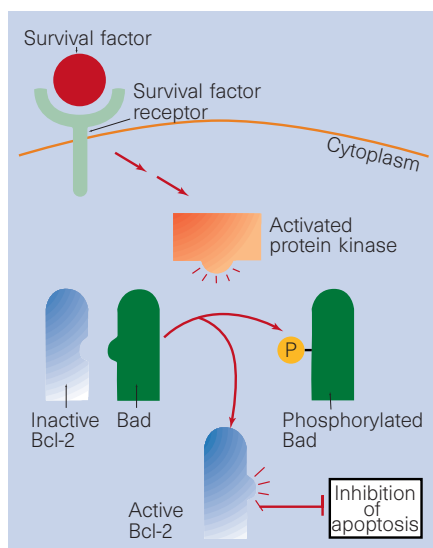


Figure 5 One way that extracellular survival factors can suppress apoptosis. The survival factor binds to a cell-surface receptor protein, thereby activating various intracellular signalling pathways. Some of these pathways lead to the activation of protein kinases that phosphorylate the Bad protein. Bad promotes apoptosis by binding to and inactivating apoptosis-inhibitor proteins such as Bcl-2. Once phosphorylated, Bad releases its bound Bcl-2 protein, which can now act to inhibit apoptosis. Thus, the survival factor suppresses apoptosis by keeping Bad phosphorylated and out of action.

gists: they not only block most forms of apoptosis in cultured cells, but also some forms of apoptosis in experimental animals. Whether they will be useful in human disease remains to be seen.

In neurodegenerative diseases, such as Parkinson's and Alzheimer's, where nerve-cell loss occurs slowly, it is still unclear if the diseased cells die by apoptosis and, if they do, whether it would be helpful to keep them alive. The finding that a viral caspase inhibitor can prevent the loss of nerve cells in the retina and maintain vision in a retinal degenerative condition in *Drosophila* provides grounds for hope³¹, especially as the fly disease closely resembles a common genetic condition (retinitis pigmentosa) in humans that causes progressive blindness. The same caspase inhibitor, however, fails to prevent nerve-cell death in a fly model of Huntington's disease³².

In cancer the goal is the opposite. One wants drugs that will activate apoptosis in tumours. Fortunately, most of the cell-killing anti-cancer drugs used today work at least partly in this way. Unfortunately, they also affect some normal cells, which is why they can have such severe side effects. The challenge remains to find ways to kill cancer cells specifically.

The death programme is often regulated abnormally in cancer cells: for instance, Bcl-2 or other apoptosis inhibitors may be

increased, or the *p53* gene may be inactivated by mutation. The death programme itself, however, never seems to be completely inactivated in cancer cells, which is both encouraging and surprising. It is surprising because cancer cells evolve by a process of random mutation and selection, so that they become progressively more malignant as they accumulate mutations that improve their ability to survive and proliferate. Mutations that inactivate apoptosis should therefore be advantageous to cancer cells, making it a mystery why the death programme always seems to be present. Perhaps it has too many lethal parts to be inactivated completely.

Questions

Despite impressive recent progress in understanding apoptosis, many mysteries remain. How do cells integrate apoptosis-promoting and apoptosis-suppressing signals to decide whether to live or die? How is the balance between cell division and apoptosis maintained in adult organs? Do animal cells also have caspase-independent suicide programmes? The crucial practical question is whether apoptosis-blockers will be useful in the treatment of human diseases. For conditions like heart attacks and strokes, where cell death is confined to hours or days, I am optimistic. □

Martin Raff is in the MRC Laboratory for Molecular and Cell Biology, and the Biology Department, University College London, Gower Street, London WC1E 6BT, UK.

e-mail: m.raff@ucl.ac.uk

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Visible viruses

The structure of viruses was for a long time an enigma. It took an amalgam of techniques, especially the rapidly burgeoning field of electron microscopy, to reveal the quasi-symmetrical nature of viral architecture.

Martin Kemp

The exciting disclosure of the polyhedral structure of viruses during the late 1950s and early 1960s presents a perfect example of collaborative interaction between different methods of experimentation, observation, analysis and visualization, at a stage when no one method could alone deliver all the answers, because each was operating at the very margins of credibility given the immense problems of concentrating and purifying viruses. Crude X-ray diffraction, chemical analysis and shadow electron microscopy, especially with plant viruses, had begun to hint at the fascinating structures that might be involved in viral architecture, but direct viewing remained frustratingly out of reach.

The introduction of the technique of negative staining in electron microscopy by Sydney Brenner and Robert Horne in 1959 opened a visual door onto the beautiful structures and symmetries displayed in the sub-microscopic world of viruses. The method involves staining of the interstices in the structure by embedding the viruses in potassium phosphotungstate, an electron-dense material. Suitably prepared, stained and mounted, the rod-like tobacco mosaic virus and the 'spherical' turnip yellow virus stole suggestively into view, regular in configuration yet tantalizingly elusive in the precise details of their structural geometry.

The technique rapidly proved its efficacy, providing ever better definition of the symmetrical properties of these and other viruses. The improved images stimulated

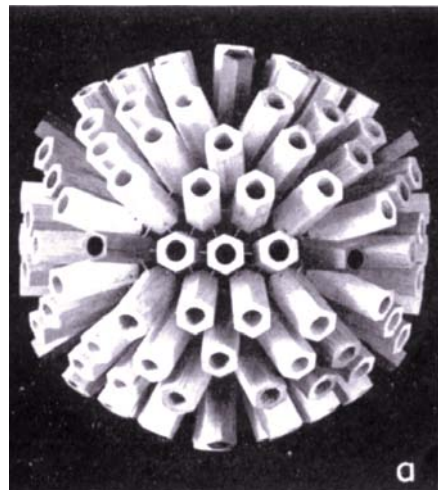


Figure 2 Wooden model of a herpes virus with 150 hexagonal and 12 pentagonal prisms. (From Fig. 8 of ref. 1.)

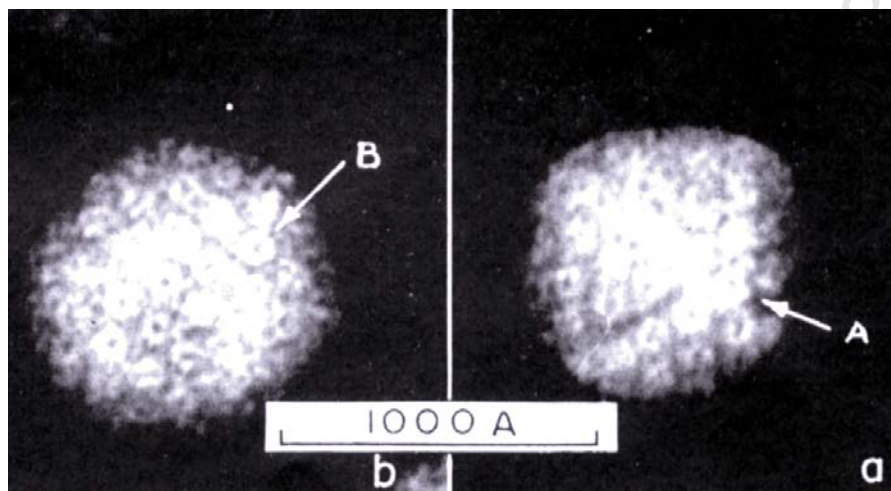


Figure 1 Electron micrograph of herpes viruses with a five-sided unit (A) and a six-sided unit (B). (From Fig. 5 of ref. 1.)

the building of three-dimensional models that were consistent with the emerging patterns — often still unresolved in their smaller details — in creative dialogue with the X-ray diffraction procedures that had originally disclosed the existence of symmetries. Working in Glasgow in 1960, Peter Wildy, Willie Russell and Bob Horne obtained particularly revealing electron micrographs of the negatively stained herpes simplex virus¹. When devoid of their floppy envelopes, the protruding prisms of the morphological units or capsomeres appeared to be arranged in the 5–3–2 symmetry of an icosahedron. The capsomeres were hexameric in cross-section, with the exception of 12 at the apices that were pentameric (Fig. 1). A neat wooden model served to demonstrate the hypothetical structure (Fig. 2).

The most extended investigation of the geometrical parameters of the architecture was subsequently undertaken by Donald Caspar and Aaron Klug, as detailed at the Cold Spring Harbor Symposium in 1962. They proposed that the icosahedral regularity of the capsid and the less regular distribution of the capsomeres sometimes revealed by the electron microscope pictures could be reconciled by the concept of quasi-symmetry. In a companion article, Wildy and Douglas Watson drew parallels with the families of polyhedra characterized by Buckminster Fuller, the American visionary architect of geodesic domes whose name is now indelibly associated with C_{60} (buckminsterfullerene). As with the modelling of C_{60} in 1985, Fuller's geodesic domes proved a vital stimulus to sci-

entific visualization. The design principles for domes, viruses and fullerenes alike combined structural economy with operational efficiency.

The story of the disclosure of the regular structure of viruses combines all the best elements of a scientific detective story. There are related bodies of visual evidence that each hover on the border of intelligibility; there is elegant speculation shaped by the search for coherent order and spiced with aesthetic intuition; and, not least, there are the empirical 'cook-book' procedures for preparation and staining to reveal the hidden secrets.

Not for nothing did Wildy and Watson quote Lewis Carroll in their contribution to the 1962 symposium:

*"You boil it with sawdust; you salt it with glue:
You condense it with locusts and tape;
Still keeping one principle object in view —
To preserve its symmetrical shape."*

And, as always, there is the question of 'whodunnit'. The suspects in this case remain, on the one hand, self-organization according to rules of mechanical necessity and, on the other, the organizing dictates of the nucleic acids. Or do they share joint responsibility? □

Martin Kemp is in the Department of the History of Art, University of Oxford, 35 Beaumont Street, Oxford OX1 2PG, UK.

e-mail: martin.kemp@trinity.oxford.ac.uk

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Glutamate-receptor genes in plants

In animal brains, ionotropic glutamate receptors (GluRs) function as glutamate-activated ion channels in rapid synaptic transmission. We have now discovered that genes encoding putative ionotropic GluRs exist in plants, and we present preliminary evidence for their involvement in light-signal transduction. It may be that signalling between cells by excitatory amino acids in animal brains evolved from a primitive signalling mechanism that existed before the divergence of plants and animals. Our findings also help to explain why neuroactive compounds made by plants work on receptors in human brains.

We isolated from *Arabidopsis* two full-length complementary DNAs, *GLR1* and *GLR2*. Each of these cDNAs encodes all of the signature domains of animal ionotropic GluRs, including the 'three-plus-one'

transmembrane domains (M1 to M4) and the putative ligand-binding domains (GlnH1 and GlnH2), previously shown to be conserved between *Escherichia coli* glutamine permease (GlnH) and animal ionotropic GluRs¹ (Fig. 1). Hydropathy plots, transmembrane prediction² and protein-sorting³ programs predict that the *Arabidopsis* *GLR* cDNAs encode a plasma-membrane signal peptide and four transmembrane domains (M1 to M4), of which M2 is predicted not to span the membrane (Fig. 1b). This membrane topology is analogous to animal ionotropic GluRs in which the putative ligand-binding GlnH domains are exposed to the external side of the membrane⁴.

In addition to their similar secondary structures, the *Arabidopsis* GLRs also share extensive sequence identity with animal ionotropic GluRs in the signature domains

GlnH1 (including the ligand-binding residue R), GlnH2, and M1 to M4, especially within M3 (Fig. 1a). The degree of identity between *Arabidopsis* GLRs and animal ionotropic GluRs within these domains (63% to 16%) is similar to that between animal ionotropic GluR subtypes that have kainate/AMPA rather than NMDA as agonists (60% to 32%). *Arabidopsis*, like animals, seems to have a family of expressed genes encoding ionotropic GluRs, as judged by the existence of other *GLR* genes (Fig. 1a) and northern blot analysis (data not shown). *GLR*-related genes also seem to exist in a variety of higher plants, including both dicotyledons (tobacco and pea) and monocotyledons (rice and maize), as judged by heterologous Southern blot analysis (not shown).

To investigate the possible *in vivo*

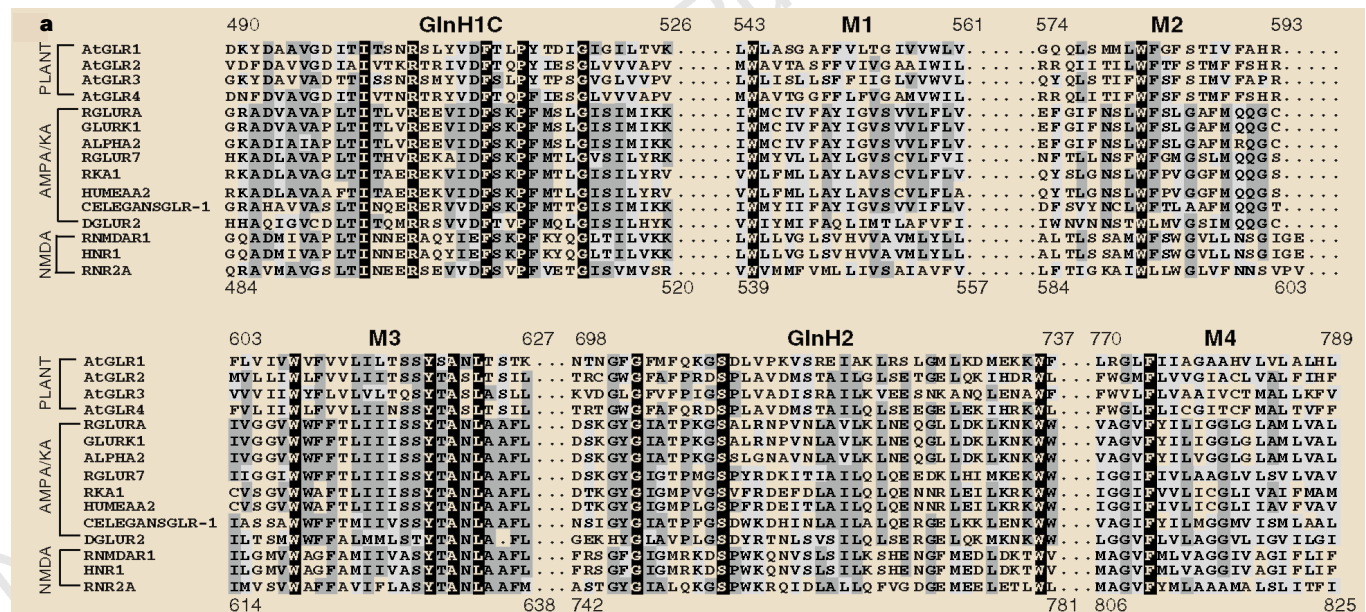


Figure 1 *Arabidopsis* *GLR* cDNAs encode proteins with sequence identity to animal ionotropic glutamate receptors. **a**, Sequence alignment of signature ionotropic GluR domains conserved between *Arabidopsis* *GLR* genes (AtGLR1-4) and animal ionotropic GluR subtypes using kainate/AMPA or NMDA as agonists. Numbers above the sequences correspond to residues of *Arabidopsis* *GLR1*; numbers underneath correspond to residues in rat *GLUR1*. Residues between the conserved domains are shown as dots. *Arabidopsis* *GLR1* (Genbank accession no. AF079998) and *GLR2* (AF079999) sequences are from full-length cDNAs obtained by screening cDNA libraries with expressed sequence tag clones (accession nos T22862 and R29880, respectively). The *GLR2* cDNA corresponds to *Arabidopsis* genomic sequence (accession no. AC002329). *GLR3* and *GLR4* sequences were derived from *Arabidopsis* genomic sequences of a bacterial and yeast artificial chromosome clones (AF007271 and AC000098, respectively). Animal ionotropic GluR genes used in the alignment are: AMPA/kainate subtypes, accession numbers M36418, X17184, X57498, M83552, X59996, S40369, U34661, M73271; or NMDA subtypes, accession numbers X63255, L05666, M91561. Sequences were aligned using Clustal X⁸.

b, Transmembrane organization of *Arabidopsis* *GLR1* based on hydropathy plot, TMPred² and PSORT³ analysis. Hydrophobic domains include a putative plasma-membrane signal sequence (hatched box) and four transmembrane domains M1-M4 (filled boxes), of which M2 is predicted not to span the membrane. Open boxes denote the two putative extracellular glutamate-binding domains GlnH1 and GlnH2 (also called S1 and S2), identified in animal ionotropic GluR genes as sharing sequence identity with *Escherichia coli* glutamine permease¹. GlnH1C denotes identity within the carboxy-terminal half of the GlnH1 domain.

function(s) of putative ionotropic GluRs in plants, *Arabidopsis* seedlings were grown in the presence of DNQX, an antagonist of animal kainate/AMPA ionotropic GluRs. Plants grown on media containing DNQX phenocopy *Arabidopsis* long-hypocotyl (*hy*) mutants impaired in light-signal transduction⁵, as judged by two independent criteria: DNQX at least partly blocks the ability of light to inhibit hypocotyl elongation (Fig. 2a–c) and to induce chlorophyll synthesis (Fig. 2d–f). Plants grown in the light in the presence of DNQX display a significant light- and dose-dependent increase in hypocotyl elongation compared with control untreated plants (Fig. 2b,c). This effect is specific for light, as the same DNQX concentration has no such effect in the dark (Fig. 2a). The increase in hypocotyl length in DNQX-treated plants (1.5- to 2-fold) is similar to that of *Arabidopsis hy* mutants

deficient in the synthesis of the phytochrome photoreceptor chromophore (*hy1* and *hy2*; 1.5- to 2.5-fold)^{5,6}.

Because light and hormones interact to control hypocotyl elongation, one or both may be responsible for the DNQX effect. We therefore measured the effects of DNQX on a second light-dependent process, chlorophyll synthesis. DNQX treatment impairs the ability of light to induce the synthesis of chlorophyll in plants grown in the dark and exposed to light for 5 hours (Fig. 2d–f). DNQX-treated plants exhibited a 60% reduction in light-induced chlorophyll accumulation (Fig. 2e) compared with controls (Fig. 2d). This effect is light specific as growth and chlorophyll levels are unaffected in DNQX-treated plants grown in the dark (Fig. 2a,f).

Taken together, these findings indicate that ionotropic GluRs may be involved in

light-signal transduction in plants. Because *Arabidopsis*, like animals, seems to possess a family of *GLR* genes, the *in planta* effects of DNQX-treated plants may be due to inhibition of one or more ionotropic GluR receptors *in vivo*.

The existence of putative ionotropic GluRs in plants is surprising, and provides an insight into why plants make chemicals that act on human brains. Several classical ionotropic GluR agonists that activate and define specific ionotropic GluR subtypes in brains are natural plant products. Examples include kainate, synthesized in the seaweed *Digena simplex*, and quisqualic acid, found in *Quisqualis indica* seeds⁷. Other less well known plant-derived ionotropic GluR agonists include β -N-methyl-amino-L-alanine, produced in cycads, and β -N-oxalylamino-L-alanine, from chick-peas, both of which are associated with neurodegenerative diseases in animals⁷.

It is traditionally believed that plants produce such neurotoxins for defence against herbivory. However, the discovery of putative ionotropic GluRs in plants and their possible role in light-signal transduction suggest an alternative theory: that plants may synthesize ionotropic GluR agonists in order to regulate their endogenous ionotropic GluR receptors, and that selective pressure in certain species then led to high-level production for defence against herbivores.

The existence of putative glutamate receptors in plants raises the possibility that other neurologically active compounds made by plants (such as nicotine, cocaine and caffeine), which act on receptors in animal brains, may also act as ligands for endogenous receptors in plants to regulate a variety of as yet unknown cell-to-cell signalling systems in higher plants.

Hon-Ming Lam*, **Joanna Chiu†**,
Ming-Hsiun Hsieh†, **Lee Meisel†**,
Igor C. Oliveira†, **Michael Shin†**,
Gloria Coruzzi†

*The Chinese University of Hong Kong,
Shatin, New Territories, Hong Kong

†Department of Biology,

New York University,

1009 Main Building, 100 Washington Square East,
New York, New York 10003, USA

e-mail: coruzzi01@mcrcr.med.nyu.edu

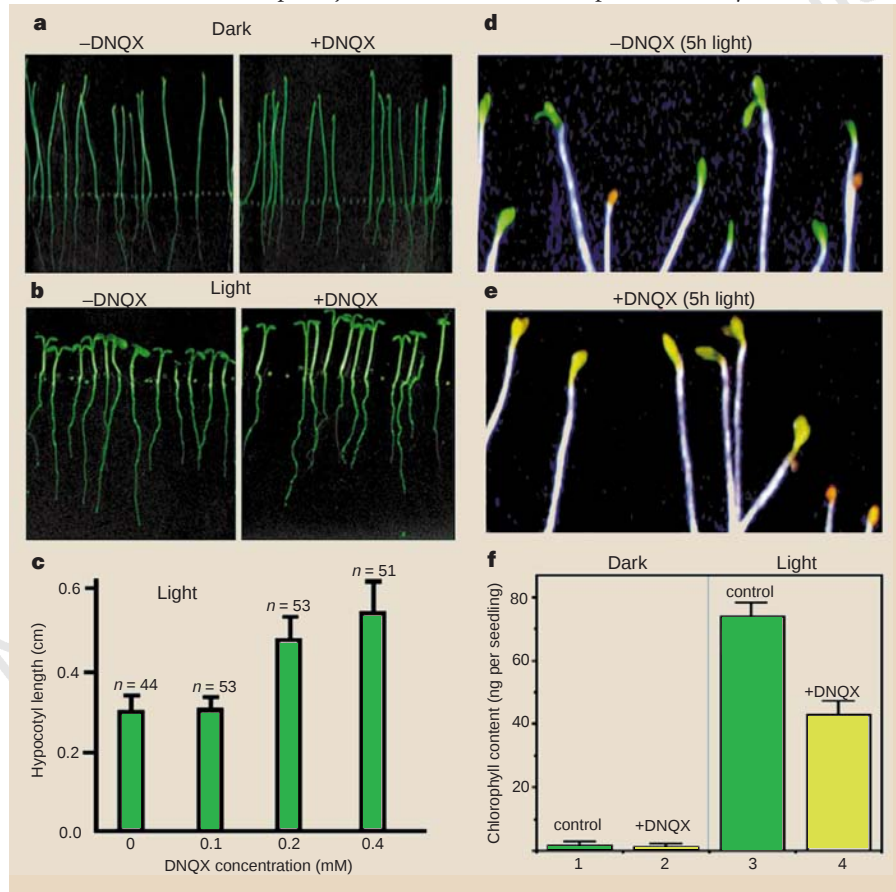


Figure 2 Effects of ionotropic GluR antagonist DNQX on *Arabidopsis* seedlings. Seedlings were sown on MS medium (Gibco BRL Murashige and Skoog basal medium containing 3% sucrose and 0.9% Difco bactoagar) in the absence or presence of the ionotropic GluR antagonist DNQX. All plates contained the solvent dimethyl sulphoxide at 1.6 μ l per ml medium. **a–c**, Effect of DNQX on hypocotyl length. Seedlings were germinated for 7 days either in the dark (**a**) or in a normal day–night cycle (16 h light: 8 h dark) (**b**) at 22 °C in the presence (400 μ M) or absence of DNQX. Hypocotyl length was determined for light-grown seedlings (n , 44–51) germinated on 0, 100, 200 and 400 μ M DNQX (**c**). An unpaired *t*-test comparing values from control and DNQX treatments: 100 μ M DNQX (no significant difference); 200 μ M DNQX ($P < 0.0001$); 400 μ M DNQX ($P < 0.0001$). **d–f**, Effect of DNQX on light-induced chlorophyll levels. Seedlings were germinated in total darkness in the absence (**d**) or presence (**e**) of 400 μ M DNQX for 7 days. Plants were transferred to light for 5 h and chlorophyll levels quantified, in three separate pools of 30–40 seedlings grown in the dark (columns 1, 2) and subsequently exposed to light for 5 h (columns 3, 4) (**f**). An unpaired *t*-test indicates a significant difference ($P = 0.0031$) in chlorophyll levels between DNQX-treated and untreated controls in light-treated plants, and no difference in dark-grown plants.

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Hotspotting called into question

Wessel and Kroenke¹ presented an age-independent geometric technique intended to refine absolute plate motions and relocate extinct hotspots. They claimed that their method indicates a recent change in the motion of the Pacific plate and relocates the Louisville hotspot to the Hollister ridge. They subsequently reported that the interpretation of raw images computed by their technique is not straightforward², but did not state explicitly how these problems affected their conclusions.

First, because the set stage pole used for computing flowlines is calculated to fit the trend of the Hawaiian–Emperor seamount chain, the clear ‘X’ on the image of cumulative volcano amplitude (CVA), which marks the location of the Hawaiian hotspot, is a normal consequence of geometry. To say that the ‘X’ illustrates the power of the technique is a tautology.

Second, Wessel and Kroenke² underestimate the effect of interference. If we plot the crustal flowline of each volcano (Fig. 1a), the biggest and brightest CVA maxima

shown in their image are obtained by the intersection of flowlines issued mainly from different alignments that are not related. To avoid these problems, flowlines must not be plotted beyond the age of volcanoes, but this seriously reduces the usefulness of the hotspot method.

Third, any non-hotspot alignment created, for instance, within a fracture zone parallel to a small circle described by the Hawaiian–Emperor chain, for instance, will converge at a single point of maximum focus. Some Pacific alignments also present two or more periods of volcanic activity^{3–5}. It is not possible to discriminate between the diagenetic volcanoes other than by measuring their age: they will contribute to the same CVA simply because they are on the same trend. Moreover, the pole reported by Wessel and Kroenke¹ fits the recent bend of the Hawaiian trend but fails to describe most Pacific alignments that are more than 5 million years old (Fig. 1b). Furthermore, it relocates the present Louisville hotspot to the Hollister Ridge, south of the Eltanin fracture zone, which is not consistent with geochemical constraints⁶.

Finally, Wessel and Kroenke¹ say that the hotspotting concept can be used to refine absolute plate motions by seeking perturbations to the initial stage poles that will focus

the CVA image. One of the problems is to define criteria that can be used to focus the image. Refining criteria based on statistics restricted to Hawaii and Louisville seamounts is not an easy task, as it is not possible to modify one stage pole and keep a good fit on both alignments without modifying the other poles.

We think these examples show that hotspotting is a flawed technique. Its application is not age-independent in practice, and it has no advantage over the classical technique of backtracking. Indeed, using the few existing dated volcanoes, it is possible to ‘pseudodate’ most Pacific volcanoes by backtracking and linking the co-genetic alignments.

**Daniel Aslanian, Louis Géli,
Jean-Louis Olivet**

*Ifremer, Marine Geosciences Department,
BP 70, 29280 Plouzané, France
e-mail: aslanian@ifremer.fr*

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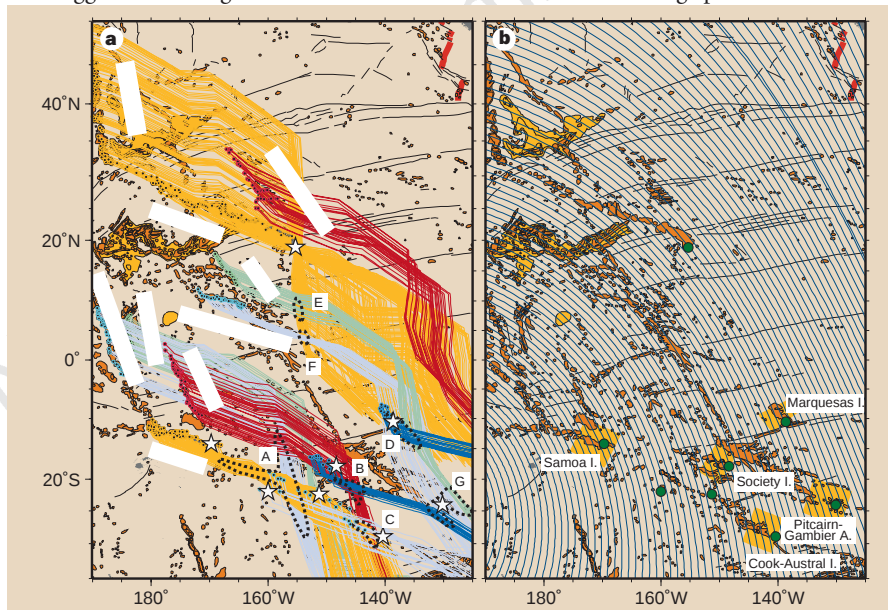


Figure 1 Analysis of the hotspotting technique. **a**, Crustal flowlines for a selection of alignments. The brightest CVA maxima (from Fig. 6 of ref. 1) are the dotted thick contours labelled A–G. The Rarotonga CVA maximum (A) is mainly the result of the intersection of crustal flowlines of the Samoa (from 10 Myr to 0 Myr ago)⁷ and the Cretaceous Marshall–Gilberts Islands (which does not present any age progression)⁸. The Mehetia CVA maximum (B) and Macdonald CVA maximum (C) are both underlined by the flowlines from the mid-Cretaceous Phoenix islands⁹. The Fatuhiva CVA maximum (D) occurs at the intersection of flowlines from the Northern Line islands, the Central Basin Ridge and the Marquesas alignment. The Emperor Chain flowlines produce two CVA maximum artefacts (E and F) with, respectively, the Northern Line islands and the Central Basin Ridge flowlines. Age and/or direction constraints demonstrate that there is no possible genetic relationship between those hotspots and the respective alignments. **b**, Small circles calculated with the most recent pole in ref. 1 (25° N, 27° W). This pole fails to describe most of the alignments less than 5 Myr old (highlighted in yellow). Instead, using conventional backtracking, we propose a pole (260° W, 57° N)¹⁰ that locates the Louisville hotspot at 50.9° S, 137.6° W, near a 0.5-Myr-old volcano¹¹ known to have a Louisville isotope signature¹². Oceanic plateaux (orange) and volcanoes (red) are digitized from the gravity map in ref. 13.

Wessel and Kroenke reply — Despite the argument of Aslanian *et al.*, hotspotting is a valid technique. It is complementary to backtracking in that it constitutes a different representation of the same hotspot–plate motion relationship in which geometry and time are separated.

By saying that the ‘X’ illustrates the power of the technique¹, we meant that it is straightforward to use undated seamounts and that the precision of the CVA maximum (the optimal hotspot location) is greatly increased relative to the large scatter of the few backtracked seamount locations. The clear ‘X’ at Hawaii is indeed a consequence of the geometry, which is also weighted by seamount sizes. Radiometric ages are required to assess age progressions, but we maintain that hotspotting is important in assessing optimal hotspot locations.

Flowlines associated with lineaments discussed by Aslanian *et al.* provide interference that is part of the broad Rarotonga CVA maximum, but large Western Pacific seamounts (not used in their Fig. 1a) also contribute. Age progression in the Western Pacific does seem to be present² and may be consistent with an origin near Rarotonga. Nevertheless, a detailed interpretation of the Pacific-wide CVA image was outside the scope of our paper, which focused on

presenting the technique and showing what could be gleaned from the Hawaii and Louisville chains. Our original CVA image¹ was derived from all Pacific seamounts, including many that are clearly not related to hotspots. A more sophisticated analysis would involve removing such seamounts and ridges, and would perhaps attempt iterative hotspotting³.

Hotspotting is blind to the ages of features. However, we have never argued that ages should not be used, but simply that hotspotting does not require them. Investigations of mid-plate tectonics would do well to use all available data and both methods.

Our ~3-Myr-old stage pole and consequent relocation of the Louisville hotspot are controversial but plausible. Hollister lava compositions represent mixtures of Louisville plume and depleted mid-ocean-ridge basalts⁴ and do not require a separate mantle source⁵. Five long-lived Pacific hotspots (Hawaii, Louisville, Cobb, Bowie and Caroline) have produced seamounts giving rise to trails (and CVA intersections) consistent with our plate-motion model. Furthermore, the short Marquesas lineament is fitted by our model only; other short-lived age-progressive chains in French Polynesia are not. Finally, the Late Neogene evolution of circum-Pacific tectonism is consistent with our model⁶.

Aslanian *et al.* are sceptical about using hotspotting to refine plate-motion models. Whether or not an inverse formulation incorporating both backtracking and hotspotting can be successfully implemented is still unresolved, but we believe it is worth investigating and that the criticism of such inversions by Aslanian *et al.* is premature.

Hotspotting is not flawed, but its success depends on assumptions that may not always hold in practice. It allows for the inclusion of much larger data sets and improves the precision of the optimal hotspot locations. Unlike 'pseudodating', it may even be applied without the *a priori* assignment of seamounts to particular chains. Like backtracking, hotspotting has both strengths and weaknesses. It is in its infancy and has been developed only as a forward method. We will no doubt learn how to use the technique more effectively as its use becomes more widespread.

Paul Wessel, Loren Kroenke

School of Ocean and Earth Science and Technology,
University of Hawaii,
1680 East-West Road, Honolulu,
Hawaii 96822, USA
e-mail: wessel@soest.hawaii.edu

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Music training improves verbal memory

Magnetic resonance imaging has shown that the left planum temporale region of the brain is larger in musicians than in non-musicians¹. If this results from a change in cortical organization^{2,3}, the left temporal area in musicians might have a better developed cognitive function than the right temporal lobe. Because verbal memory is mediated mainly by the left temporal lobe, and visual memory by the right^{4,5}, adults with music training should have better verbal, but not visual, memory than adults without such training. Here we show that adults who received music training before the age of 12 have a better memory for spoken words than those who did not. Music training in childhood may therefore have long-term positive effects on verbal memory.

To determine the effect of neocortical development associated with music training, we studied the verbal and visual memories of a group of adults. We studied 60 female college students from the Chinese University of Hong Kong, of whom 30 had had at least six years of training with a Western musical instrument before the age of 12, and 30 had received no music training. The two groups of participants were matched ($P > 0.01$) in terms of age (music training, 19.9; no music training, 19.6; $t = 0.99$), grade point average (music training, 3.0; no music training, 3.0; $t = 0.21$) and years of education (music training, 14.7; no music training, 14.3; $t = 2.07$).

We assessed the verbal memory of each subject by the number of words she could

recall in a list-learning task in which a 16-word list was presented orally three times to each subject. After each presentation, the subject was required to recall as many words as she could. We evaluated each subject's visual memory by the proportion of ten simple figures that she could draw from memory in the Benton visual-retention test⁶.

We found that adults with music training learned significantly more words than those without any music training ($F(1,58) = 17.69$, $P < 0.01$); results were consistent across the three trials ($F(2,116) = 274.05$, $P < 0.01$; Fig. 1). However, the performances of adults with (score: 7.2 out of 10) and without (score: 6.9) music training were not significantly different in the visual memory task ($t(58) = 1.00$, not significant). Both results were replicated when the subjects were tested again with another list-learning task ($t(26.89) = 3.07$, $P < 0.01$) and another visual-memory task (the Rey-Osterreith⁷ figure immediate recall task; $t(26.88) = 0.44$, not significant).

Because most memory-training programmes are based on mnemonic and compensatory techniques⁸, the idea of using music training to improve verbal memory seems unusual. However, music training has advantages over other techniques. First, it may be easier to engage children in playing musical instruments, which is an enjoyable activity, than in mnemonic strategies. Second, musical training requires little verbal skill, so it may be more suitable as a memory-training technique for patients with language impairment.

Our results provide preliminary evidence that music training may have a long-term effect on the improvement of verbal memory. Investigation of the effects of the age at which music training begins, and of the duration of training, will provide corroboration and theoretical refinement of our results.

**Agnes S. Chan, Yim-Chi Ho,
Mei-Chun Cheung**

Department of Psychology,
The Chinese University of Hong Kong,
Shatin, Hong Kong, China
e-mail: aschan@psy.cuhk.edu.hk

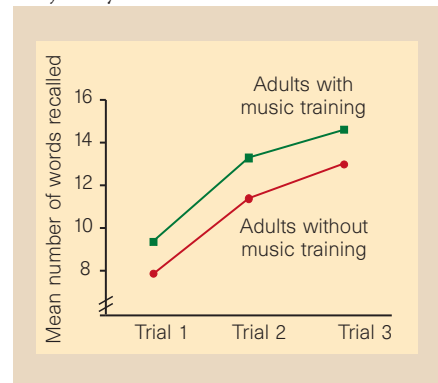


Figure 1 Mean number of words recalled by adults with and without music training. The list-learning task consisted of 16 words from four categories ('family member', 'country', 'furniture' and 'vegetable') that were presented in a fixed random order. The list was presented orally three times to each subject, who was asked to recall as many words as possible after each presentation. The subjects with music training recalled on average 16% more words than those with no music training.

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How many more eureka's in the bath?

What Remains To Be Discovered: Mapping the Secrets of the Universe, the Origins of Life, and the Future of the Human Race

by John Maddox

Free Press/Macmillan: 1998. 433 pp. \$26, £20

Carl Zimmer

One can only imagine how annoyed John Maddox must have become in 1996. He had retired from *Nature* a year earlier, after an impressive 23-year stint as its editor. Having successfully guided this journal into the age of space-based telescopes and genome projects, he decided it was a good time to write a book. There he would lay out the most important questions still left unanswered by scientists. And then, just as he was getting elbow-deep in the project, everyone suddenly started talking about another book on what the future will bring: John Horgan's *The End of Science* (Helix, 1996).

Horgan, a journalist, had interviewed leading scientists and strung profiles of them together into an argument that science as we've known it in the past few centuries — unveiling the Universe's fundamental secrets — is coming to a close. Much of the important stuff, such as relativity and DNA, has already been discovered, and the things that haven't, Horgan claimed, are either minor details or dreamy notions beyond our powers to verify. Many scientists loathed the book, and Maddox himself wrote in a review that it was "intelligent but perverse".

Now, two years later, we have *What Remains To Be Discovered*. Nowhere does Maddox mention Horgan by name, and yet Maddox often addresses him implicitly. When Maddox writes, "Who, now, dares say that the days of surprise are over?" it is hard not to imagine Horgan lurking in his mind.

Maddox's book, I suspect, is all the better for it. He has done an excellent job of showing just how many surprises we may have in store. He divides his book into surveys of physics, cosmology, evolution, cell biology, consciousness, mathematics, and what could be called the science of calamity — global warming and other threats to humanity's future. He runs through the histories of these fields, often with great clarity. (Now at last I understand how virtual photons transmit forces between particles.) Then Maddox dives into the gaps still left after all those centuries of work. The biography of the Universe is still a shambles, despite all that's been learned since Edwin Hubble saw stars fleeing away from us. Physics still awaits the union of gravity and quantum mechanics. No one has yet truly described thought.

Although a physicist by education, Maddox is a great intellectual omnivore, and he

can recognize how different fields face similar challenges. Biologists who are trying to use genes to assign dates to the origin of humans and other lineages are like cosmologists searching for standard candles to measure the distance to stars and galaxies. Cell biologists won't be able to transcend the heaps of data they've collected until they follow the example of atmospheric scientists, who are learning how to model the world's climate on computers.

I was disappointed, though, that some important subjects warranted little or no space. I looked in vain for what remains to be discovered by ecologists (the role of species diversity in ecosystems, for example, or the control exercised on food webs from the top or the bottom). The atmosphere and the oceans only turn up in Maddox's discussion of global warming, which is a bit like saying that physics is only interesting in connection with radioactive waste.

When Maddox talks about evolution, he skips over the ongoing revolution in phylogenetic systematics. He is rightly sceptical about the claims of evolutionary psychology, but his interest in the evolution of animal behaviour seems to stop with William Hamilton's work in the 1960s. In fact, it is now a burgeoning field, with many questions still unanswered.

And Maddox never quite vanquishes his tacit foe. Horgan's book doesn't work as a coherent argument because he sets up the rules of the game so that he can't lose. If an old scientist declares that he and his generation solved all the great problems 50 years ago, Horgan wins. If another says that the ultimate answers will always remain just out of reach of the latest theory, Horgan wins. If another says that his field has ground to a halt because he and his colleagues can't figure out

how to proceed, Horgan wins. But, although it may fail as an argument, Horgan's book is interesting as a collection of questions about the nature of science. Can a science run its course, and then run out? Are there some things we can't know? What then?

Maddox hardly addresses the possibility that there are practical and theoretical limits to science. A long list of unanswered questions isn't sufficient. Most of the ones that Maddox presents won't supplant current paradigms in the way that relativity engulfed Newton's laws of motion. On the other hand, the union of quantum mechanics and gravity could indeed change the way we view the Universe but, if it requires atom smashers the size of galaxies to be tested, will it be anything more than a thought experiment?

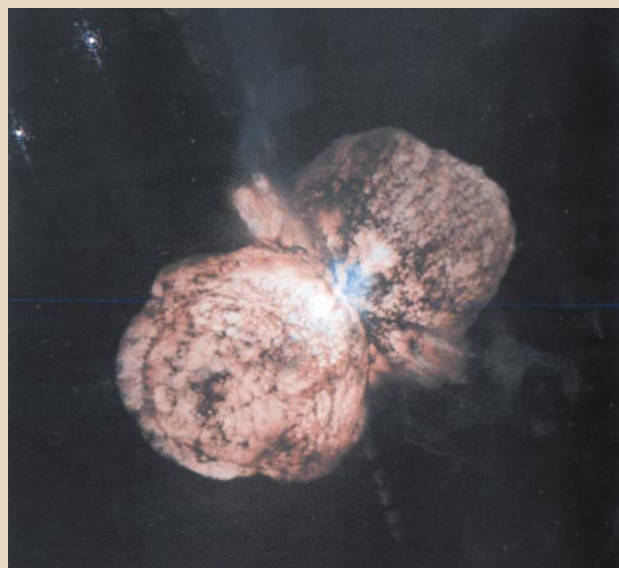
Instead of confronting these matters head on, Maddox simply asserts that science is like a set of *matryoshka*, those nested Russian dolls, with another doll always hidden inside waiting to be unscrewed. He believes this because in the past there has always been another doll. But when I unscrew a set of *matryoshka*, I know that history isn't a guarantee that the future will be like the past. I may twist one open and find nothing inside, or simply be unable to twist it because it's too small for my fingers.

I won't dare to guess whether Maddox's dolls will keep coming unscrewed or not, but I suspect that, even if they don't, science will go on with a long happy life. Natural selection doesn't automatically predict mastodons and flying fish. Quantum mechanics doesn't automatically put the Moon in the sky. There's a lot in between, and a lot to keep future editors of *Nature* working late into the night. □

Carl Zimmer is at *Discover Magazine*, 114 Fifth Avenue, New York 10011, USA.

Hubble vision

The star Eta Carinae exploded 150 years ago, since when its impressive gas nebula (right) has been expanding. This photo is from *Hubble Revisited: New Images from the Discovery Machine* by Daniel Fischer and Hilmar Duerbeck (Springer, \$40, £24.50). The book, a sequel to *Hubble: A New Window to the Universe*, contains many of the latest images from the space telescope.



Governing talk

The Word on the Street: Fact and Fable about American English

by John McWhorter

Plenum: 1998. 291 pp. £16.94, \$27.95

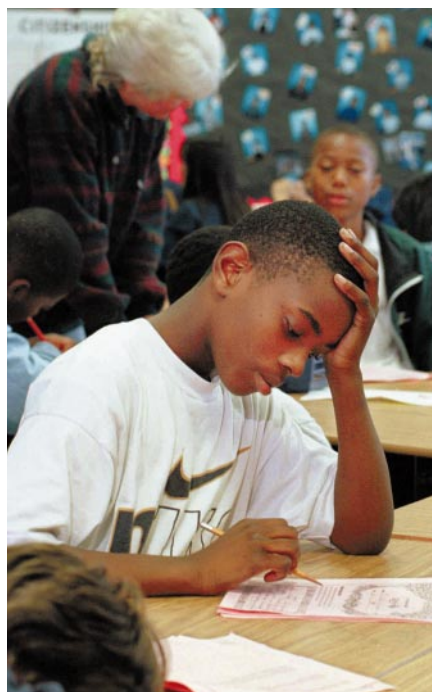
Elaine Showalter

In December 1996, the school board of Oakland, California made national headlines by declaring that 'Ebonics' — Black English — was the primary language of African-American children, and that they would study English as a foreign language. The board's resolution relied on studies claiming that Black English was a "genetically based" African language system, rather than a dialect of American English. One proponent of Ebonics, Ernie Smith, argued that African-American children do poorly in school because they are "west and Niger-Congo Africans in diaspora". Using Ebonics as a bridge to standard English, the board hoped, would build racial pride and improve learning.

Reaction to the decision was predictably heated, with objections from white educators, parodies by journalists such as Chicago's Mike Royko ("I be tired hearing abouts Ebonics. I dislike hearin about it no more"), and calls for the black community to "speak out against Ebonics" from the National Head Start Association, whose intentionally startling newspaper advertisements showed Martin Luther King saying "I HAS a Dream" (see *Nature* 386, 321–322; 1997).

John McWhorter, linguistics professor at the University of California at Berkeley, was one of the few black scholars who openly disagreed with the board's recommendations and with its claims about language. A specialist in creole and pidgin languages, he has now written a fascinating and provocative book to place the Ebonics controversy in the context of an evolutionary theory of language in general and American English in particular. All languages change and evolve; at any moment they are a "bundle of dialects" which cross-fertilize each other; and grammatical and verbal uses are no more innately 'correct' than a giraffe or the coming of spring is 'correct'. Drawing on an impressive knowledge of the systems of the 5,000 languages now spoken in 170 countries, McWhorter demonstrates that "languages, like ovens, are self-cleaning" — that linguistic innovation does not survive if it impedes communication. Black English is a dialect, not a language, he argues, and indeed is less remote from standard English than the dialects of many linguistic systems.

In the second part of his book, McWhorter tackles three current issues to elaborate his point: prescriptive grammar, which forces people to retain outmoded usages such as "whom"; feminism and the gender-neutral pronoun; and the relevance of Shakespeare



Classroom controversy: Black English is a dialect, not a distinct language, argues McWhorter.

to American audiences. He is funniest and most outrageous on the Bard, declaring that "while I have enjoyed the occasional Shakespeare performance and film, most of them have been among the dreariest, most exhausting evenings of my life — although only recently have I begun admitting it". Shakespeare is incomprehensible to contemporary American audiences because his language is as archaic as Chaucer's. Everyone would benefit, McWhorter believes, from high-quality modern English translations. Then people would flock to see *King Lear*, and directors would not have to concoct bizarre productions to achieve relevance. After all, McWhorter persuasively notes, the non-English-speaking world has had to read Shakespeare in translation all along.

The argument is not new; critics have suggested it for at least a century, and Shakespearean scholar Maurice Charney revived it a decade ago. But McWhorter's argument undercuts his own theory that language evolves in the human marketplace of communication. After all, Shakespeare is out of copyright, and anyone who wants to produce a modernized version is free to do so. They don't, however, because successful contemporary versions of Shakespeare either transpose the plot and not the language, as in *West Side Story*, or create strong visual elements to clarify language that is poetic and strange but not incomprehensible, as in the Leonardo DiCaprio film *Romeo and Juliet*. If Shakespeare survives, he be doing jus fine.

In the final section of the book, McWhorter demolishes the case for Black English as an African language, showing that it has British rather than African linguistic

origins, and proving it is not a remnant of the slave-dialect Gullah. And he explains that speakers of Black English do a lot of "code switching" between local and standard English forms. The real explanation for the "shockingly poor performance of black students", he argues, is "an alienation from education that is prevalent in the African-American population", and fuelled not only by frustration and rage against poverty and racism, but also by self-destructive adherence to an outmoded victimology.

McWhorter recommends a complex set of educational changes. First, accept Black English as a legitimate and creative dialect, rather than a stigmatized one, and allow young children to speak in their home dialect in the classroom. Teach the African-American cultural heritage alongside others in the American tradition. But teach all children to read in standard English. Do not provide Ebonics readers; immerse children in the language of the mainstream while acknowledging that Black English itself is changing standard American English day by day, as anyone who has teenagers or lives in a city can vouch. The word on the street may eventually be the word in the lecture hall or the senate. And linguistic history shows that the language that survives is going to be the most vivid, economical — and fun. □

Elaine Showalter is in the Department of English, Princeton University, Princeton, New Jersey 08544, USA.

Eat or be eaten

The Raptor and the Lamb: Predators and Prey in the Living World

by Christopher McGowan

Penguin: 1998. 270 pp. £18.99, \$25

John R. G. Turner

The most striking event of our vacation this summer was the morning we looked out of our window to see one of "our" half-tame greater spotted woodpeckers being eaten by "our" not quite so tame resident pine marten. The world, said Erasmus Darwin, "would seem to be one great slaughterhouse . . . whose first law might be expressed in the words 'eat or be eaten.'" *The Raptor and the Lamb* is an eloquent exposition of the consequences of being somewhere in the food chain.

The heart of the book is a series of loving accounts, written in a plain prose that verges on poetry, of the behavioural, physiological and anatomical adaptations of predators for the capture of prey, and of the prey for the avoidance of capture. The *pas de deux* of the hunter and the hunted, whether in the unfamiliar world of the blind copepod, a minute crustacean, finding its prey by sensing "near-field displacements" in the water, or the more familiar world of the zebra throttled by



The killing fields: African wild dogs attack a wildebeest in Tanzania.

the lion, is analysed in a style that makes for compulsive reading. Christopher McGowan clearly loves animals and their adaptations, and this is a thoroughly enjoyable popular account, written for the reader who likes something meatier than a TV wildlife spectacular. The illustrator, Abigail Rorer, deserves a better billing for her charming line drawings than the admittedly handsome mention in the acknowledgements.

Vertebrate predators take the lion's share of the text: mammals, birds, reptiles and sharks, hunting by sea, air and land. Comparative morphology and physiology are more fun in this book than I could ever have believed; they are then applied to make some excellently cautious and reasonable reconstructions of the predatory interactions of dinosaurs, and a wonderful foray into the different sensory world of the predatory planktonic crustacea. The text throughout is punctuated with visually vivid vignettes describing individual acts of attack and destruction.

I am intrigued by McGowan's apparent rejection of the concept of a co-evolutionary "arms race" between the prey and the predator. The adaptations of the predators, he makes it very clear and in elegant detail, have evolved for the apprehension of their prey. He proposes however that the evolution of counter-strategies by the prey does not *in itself* cause any further evolution by the predator. The running abilities of African herbivores are not a response to their being hunted, but are an adaptation for travelling long distances in search of grazing. The gazelle's ability to sprint from a cheetah is merely a by-product of its need to run efficiently in search of grass. But using an analogous argument, I can challenge McGowan's belief that moths which mimic butterflies have been forced to become diurnal, to fly

with their model. Surely, it is moths that are *already* diurnal that evolve mimicry.

The chapters on camouflage, natural chemical warfare, and plant defences through trichomes (their surface outgrowths) and secondary compounds, are good, but less compelling. McGowan is at his best with the sheer physicality of hunting: the magnificence of top predators such as falcons, lions, cheetahs and hunting dogs, or the effects of viscosity on motion at the scale of the plankton. In its objectivity and refusal to be emotional, the text becomes very moving — for example, in the account of "suicide" by an African buffalo cornered by five lions. The spectre of sudden or more often lingering death haunts the pages through to the eloquently understated final vignette, where, in case you thought it was safe to go into the garden, *Homo sapiens* appears as a surprising victim of the eternal struggle. □

John R. G. Turner is in the School of Biology, University of Leeds, Leeds LS2 9JT, UK.

Good in parts

The Floating Egg: Episodes in the Making of Geology

by Roger Osborne

Jonathan Cape: 1998. 372 pp. £15.99

H. S. Torrens

This book can best be described as a chronological anthology, linked by Roger Osborne's commentaries, of how geological science developed in an eastern part of the largest English county — Yorkshire. If I was reviewing for the *North East Yorkshire Naturalist* (a mythical journal like much of this book's content), I might be kinder to it. But geology is a global science, and its history equally so, and to be told that much of it was invented or

discovered in Yorkshire, including stratigraphy and biostratigraphy, without any discussion of the historical situation of just these aspects of geology in other parts of England, let alone Europe or South America, is to distort that history.

The episodes of the book's subtitle were inspired by a 1984 paper cataloguing the discovery of fossil marine reptiles in Yorkshire, mainly in the Lower Jurassic Alum Shales, the deposit that inspires the first chapter, a rambling excursion into chemistry and the alum maker's art. This is where the alum maker's 'floating egg' of the book's too cryptic title appears. Only this title appears on the spine, so future second-hand book hunters may have to seek this book on the cookery shelves. The 1984 paper, by Michael Benton and Michael Taylor, documented the finds of these fossil reptiles and their subsequent careers in museums around the world. This led Osborne to the more original parts of his book, the ten chapters on each of the ten reptiles found in these shales or the underlying jet rock between 1758 and 1960.

If the book's egg seems of a highly curatic variety, at least it gives some insights of real curatorial significance. Osborne's record of the fates of the fourth reptile, a plesiosaur found some time before 1828 but now lost, the seventh, "the most magnificent", which went to Ireland, or the 2,000-mile separation of head and body of the ninth, which went to America, is both welcome and salutary. But whether these really were the first, second and so on from this part of Yorkshire is another matter. The man who coined the term 'dinosaur', Richard Owen, told the King of Saxony in 1844 that in the 30 years since the first ichthyosaur had come to scientific attention (in Dorset), at least 1,000 had been found in England alone.

The least successful parts of this book are the fictionalized chapters, which are full of fictional (or erroneous) detail. The first of these misleads about how William Smith supposedly invented stratigraphy atop York Minster in 1794. This chapter's narrator, Samborne Palmer (whose Christian name is misspelt), then receives letters from a surgeon who had been dead for ten years. In a final absurdity, Palmer describes events that happened 17 years after his own death. Apart from such fictions, we are also given much hindsight history, with Smith observing rocks in Yorkshire in 1794 that he had not yet identified. In a later, equally Smith-inspired chapter, the poor man is committed to prison a year too late. In sustained defiance of Osborne's assertion that "everything in this book is true", further errors abound.

Many more genuine episodes in the history of geology in Yorkshire go unmentioned. Peter Murray's involvement with the fossil plants of Gristhorpe Bay is one example. Another relates to the Rev. Frederick

Kendall, the first person to describe the fossils of Scarborough. He had to do so anonymously, after being sent down from Cambridge University for multiple arson attacks on his old college. His father hired the best lawyers and got him off in the lawsuit that followed, but his college knew he was guilty.

This book has, in my opinion, been inspired by the success of Richard Fortey's fine *The Hidden Landscape* from the same publishers in 1993 (reviewed in *Nature* 368, 366–367; 1994). Sadly any comparison stops there. Some of the photographs in this one are badly reproduced, the index is inadequate and much of the history it records is parochial, myopic and often factually wrong. The history of geology is no more a field for ill-informed enthusiasm than is brain surgery. □

H. S. Torrens is in the Department of Earth Sciences, Keele University, Staffordshire ST5 5BG, UK.

Written by the right hemisphere

Of Two Minds: The Revolutionary Science of Dual-Brain Psychology

by Fredric Schiffer
Free Press: 1998. 239 pp. \$25

Chris McManus

As empirical scientists, we all believe in experiments, so let's try the following. Close your left eye and cover the inside (nasal) half of your right eye. Look around using just the outer (temporal) half of your right eye. How does it feel? Now try it the other way, using the temporal half of your left eye. Does it feel any different? To be more specific, with one side did you feel distressed and immature, while with the other you smiled, and felt comforted and mature?

When Fredric Schiffer did this "[he] knew that it would be profoundly important ... [he] rigorously studied it ... [and found it had] significance for both understanding our human mind and furthering our ability to deal with a variety of emotional or psychological problems". Certainly his theory is broad. Not only does it explain and indicate the treatment for pre-menstrual syndrome, manic depression, multiple personality disorder, anxiety disorders, addictions, hypnosis, psychosis and heart disease, it will also help us be "truly healthy", and "contribute in our attempt to make this human endeavour succeed, to permit our species to achieve its full meaning and its magnificent potential".

The rationale of the experiment is that it restricts visual input to just one hemisphere, which responds with its own personality, perhaps previously suppressed. Your unconscious, immature right brain should have expressed itself when looking to the left side,

and your mature self when looking to the right.

But maybe you didn't see the world as distressing when viewed with the right hemisphere. A series of non-falsifiability gambits then follows. If the effect was reversed, you should look for hidden traumas in early life. If both views seemed equivalent, then perhaps both sides of the brain are equally calm and healthy (or equally troubled and distressed). Or maybe one side is totally dominant (although six months of therapy might help "the subordinate hemisphere become more liberated").

Schiffer's theory is an extension of hemisphericity, the dubious concept that spawned books such as Betty Edwards's *Drawing on the Right Side of the Brain* (J. P. Tarcher, 1989) and Carol G. Wells's *Right-Brain Sex* (Avon Books, 1989), which claimed that the separate processing modes of the left and right cerebral hemispheres, analytic and intuitive, could be switched on and off at will. The novelty here is that hemisphericity is taken to its ultimate conclusion, so that, "we are of two minds [and personalities], each associated with one cerebral hemisphere". In particular the "unconscious is an intact mind (albeit immature), with its own thoughts, feelings and actions, a mind we physically associate with one of the cerebral hemispheres". In true psychoanalytic fashion, this is a world of titanic struggles, "between the orbital frontal cortex (and the hippocampus), trying to calm the amygdala on each side, and ... between the entire left mind and the entire right mind". Perhaps inevitably, this version of Freud's

Project for a Scientific Psychology dressed in modern neuroscience ends in tears, mostly on the couch of the therapist, whose clinical dialogues sound like a Hollywood scriptwriter's fantasies: "At that point I explained my hypothesis because I didn't feel I could wait a few years for her to come to it in her own time ... To Carol's surprise, her symptoms suddenly abated."

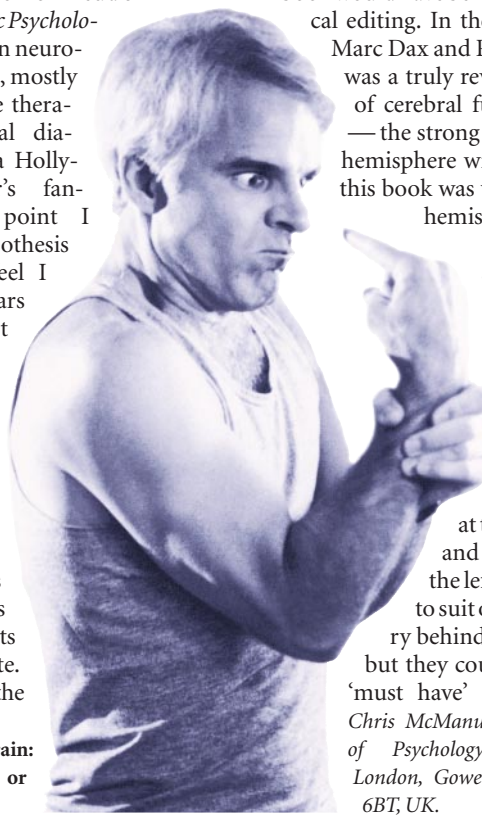
The problems of the theory are manifold. Absence of compelling empirical evidence is one, with tedious clinical transcripts being no substitute. More theoretically, the

Left brain v. right brain: respectable theory or Hollywood fantasy?

visual field restrictions don't prevent information from the fovea reaching both hemispheres, and corpus callosum transfer means any remaining asymmetries are likely to be extremely small. Particularly problematic is the specific claim that, "I found no significant differences between right- and left-handers in ... the side of their emotional responses ... and I have observed no differences in their clinical responses to therapy". If any reliable phenomenon were truly related to functional hemispheric asymmetry then differences between right- and left-handers would be a strong expectation, 2–5 per cent of right-handers and 30–35 per cent of left-handers showing atypical lateralization. Absence of an association points the explanatory finger strongly at placebo effects based on a mere metaphor. When I say I am in two minds, I do not literally mean so.

The writing style is execrable (and the diagrams no better, the anatomy of the visual tracts and half-fields being wrong). Much of the book is written in the breathless first-person historic beloved of bad popular science writing (to parody, "I am in Los Angeles. I watch the scalpel slice into the glistening pink brain ..."). Poor analogies abound, such as an image being sent through the corpus callosum, "sort of like e-mail". Peculiarly infuriating is prefixing descriptions of scientists with "prominent", "distinguished", "renowned", "very distinguished", "consummate", "highly regarded", "fine", "eminent" and "pioneering"; even the author's siblings are an "esteemed philosopher" and an "esteemed Shakespearean scholar" — the book would have benefited from their critical editing. In the nineteenth century, Marc Dax and Paul Broca made what was a truly revolutionary discovery of cerebral functional asymmetry — the strong association of the left hemisphere with language. Perhaps this book was written with the right hemisphere.

If *Of Two Minds* has any lasting impact, it may be in Schiffer's novel sunglasses idea. These 'John Lennon' specs have round lenses, with each lens completely clear at one edge and shading to dark at the other. Rotate them and have, say, input to just the left or just the right side, to suit one's mood. The theory behind them may be flawed, but they could still be next year's 'must have' fashion accessory. □
Chris McManus is in the Department of Psychology, University College London, Gower Street, London WC1E 6BT, UK.



RONALD GRANT

The genome sequence of *Rickettsia prowazekii* and the origin of mitochondria

Siv G. E. Andersson*, Alireza Zomorodipour*, Jan O. Andersson*, Thomas Sicheritz-Pontén*, U. Cecilia M. Alsmark*, Raf M. Podowski*, A. Kristina Näslund*, Ann-Sofie Eriksson*, Herbert H. Winkler† & Charles G. Kurland*

* Department of Molecular Biology, University of Uppsala, Uppsala S-75124, Sweden

† Department of Microbiology and Immunology, University of South Alabama, Mobile, Alabama 36688, USA

We describe here the complete genome sequence (1,111,523 base pairs) of the obligate intracellular parasite *Rickettsia prowazekii*, the causative agent of epidemic typhus. This genome contains 834 protein-coding genes. The functional profiles of these genes show similarities to those of mitochondrial genes: no genes required for anaerobic glycolysis are found in either *R. prowazekii* or mitochondrial genomes, but a complete set of genes encoding components of the tricarboxylic acid cycle and the respiratory-chain complex is found in *R. prowazekii*. In effect, ATP production in *Rickettsia* is the same as that in mitochondria. Many genes involved in the biosynthesis and regulation of biosynthesis of amino acids and nucleosides in free-living bacteria are absent from *R. prowazekii* and mitochondria. Such genes seem to have been replaced by homologues in the nuclear (host) genome. The *R. prowazekii* genome contains the highest proportion of non-coding DNA (24%) detected so far in a microbial genome. Such non-coding sequences may be degraded remnants of 'neutralized' genes that await elimination from the genome. Phylogenetic analyses indicate that *R. prowazekii* is more closely related to mitochondria than is any other microbe studied so far.

The *Rickettsia* are α -proteobacteria that multiply in eukaryotic cells only. *R. prowazekii* is the agent of epidemic, louse-borne typhus in humans. Three features of this endocellular parasite deserve our attention. First, *R. prowazekii* is estimated to have infected 20–30 million humans in the wake of the First World War and killed another few million following the Second World War (ref. 1). Because it is the descendent of free-living organisms^{2–4}, its genome provides insight into adaptations to the obligate intracellular lifestyle, with probable practical value. Second, phylogenetic analyses based on sequences of ribosomal RNA and heat-shock proteins indicate that mitochondria may be derived from the α -proteobacteria^{5,6}. Indeed, the closest extant relatives of the ancestor to mitochondria seem to be the *Rickettsia*^{7–10}. That modern *Rickettsia* favour an intracellular lifestyle identifies these bacteria as the sort of organism that might have initiated the endosymbiotic scenario leading to modern mitochondria¹¹. Finally, the genome of *R. prowazekii* is a small one, containing only 1,111,523 base pairs (bp). Its phylogenetic placement and many other characteristics identify it as a descendant of bacteria with substantially larger genomes^{2–4}. Thus *Rickettsia*, like mitochondria, are good examples of highly derived genomes, the products of several types of reductive evolution.

The genome sequence of *R. prowazekii* indicates that these three features may be related. For example, prokaryotic genomes evolving within a cell dominated by a much larger, eukaryote genome and constrained by bottle-necked population dynamics will tend to lose genetic information^{12,13}. Predictable sets of expendable genes will tend to disappear from the prokaryotic genome when they are made redundant by the activities of nuclear genes. Likewise, non-essential sequences and otherwise highly conserved gene clusters may be obliterated by deleterious mutations that are fixed in clonal parasite or organelle populations because they cannot be eliminated by selection. This process is ongoing in the *Rickettsia* genomes, as shown by the identification of sequences that have recently become pseudogenes. Also, a large fraction (~25%) of non-coding sequences in this genome may be gene remnants that have been

degraded by mutation and have not yet been removed from the genome. Finally, transfer of genes from a mitochondrial ancestor to the nucleus of the host would both reduce the mitochondrial genome size and stabilize the symbiotic relationship. Phylogenetic reconstructions that identify genes in the *Rickettsia* genome as sister clades to eukaryotic homologues found in the nucleus or the organelle support this interpretation. *Rickettsia* and mitochondria probably share an α -proteobacterial ancestor and a similar evolutionary history.

General features of the genome

The circular chromosome of *R. prowazekii* strain Madrid E has 1,111,523 bp and an average G+C content of 29.1% (Figs 1, 2). The genome contains 834 complete open reading frames with an average length of 1,005 bp. Protein-coding genes represent 75.4% of the genome and 0.6% of the genome encodes stable RNA. We have assigned biological roles to 62.7% of the identified genes and pseudogenes; 12.5% of the identified genes match hypothetical coding sequences of unknown function and the remaining 24.8% represent unusual genes with no similarities to genes in other organisms (Table 1). Multivariate statistical analysis has shown that there is no major variation in codon-usage patterns among genes that are expressed in different amounts, indicating that codon-usage patterns in *R. prowazekii* may be dominated mainly by mutational forces¹⁴. G+C-content values at the three codon positions average 40.4, 31.2 and 18.6%, and these values are similar at different positions in the genome. We classified the open reading frames with significant sequence-similarity scores to gene sequences in the public databases into functional categories (Table 1) that allow comparisons with the metabolic profiles of other bacterial genomes^{15–23}.

Non-coding DNA. The coding content of previously sequenced bacterial genomes is, on average, 91%, ranging from 87% in *Haemophilus influenzae* to 94% in *Aquifex aeolicum*. In comparison, a large fraction of the *R. prowazekii* genome, 24%, represents non-coding DNA (Fig. 3). A small fraction of this corresponds to

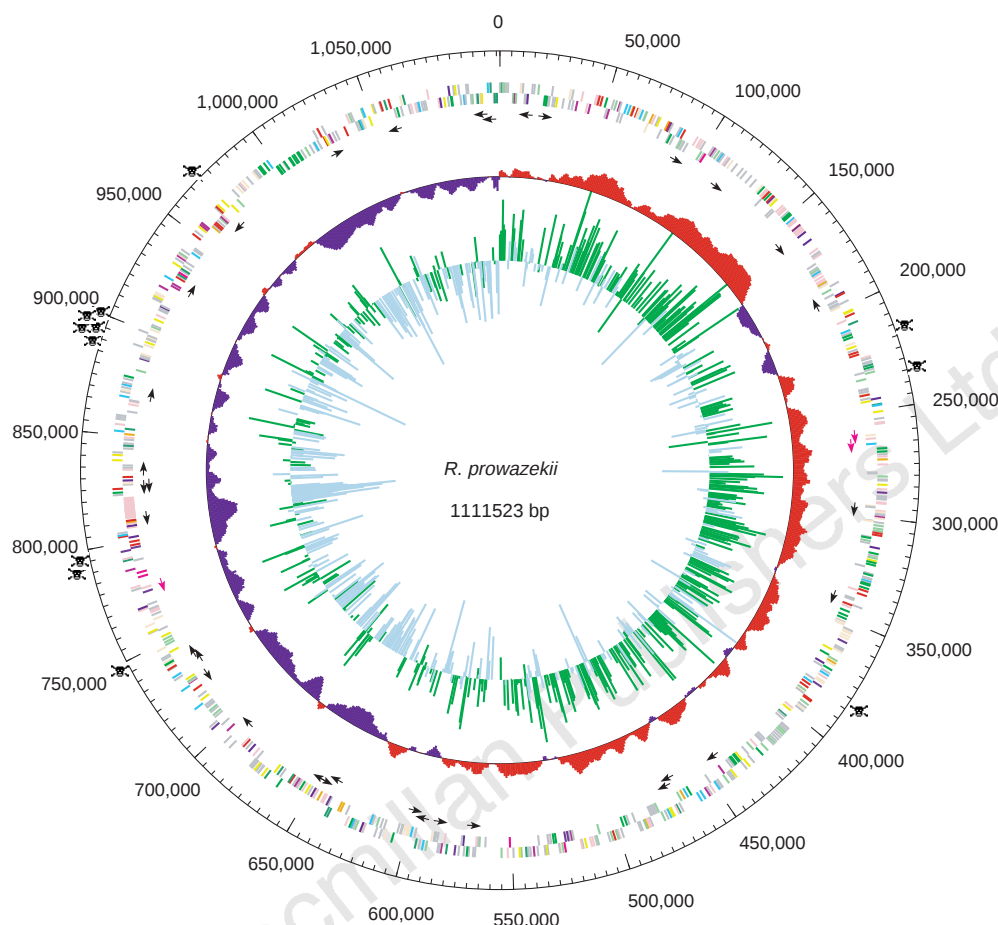


Figure 1 Overall structure of the *R. prowazekii* genome. The putative origin of replication is at 0 kb. The outer scale indicates the coordinates (in base pairs). The positions of pseudogenes are highlighted with death's heads. The distribution of genes is shown on the first two rings within the scale. The location and direction of transcription of rRNA are shown by pink arrows and of tRNA genes by black arrows. The next circle in shows GC-skew values measured over all bases in the genome. Red and purple colours denote positive and negative signs, respectively.

pseudogenes (0.9% of the genome) and less than 0.2% of the genome is accounted for by non-coding repeats. The remaining 22.9% contains no open reading frames of significant length and it has the low G+C content (mean 23.7%) that is characteristic of spacer sequences in the *R. prowazekii* genome¹⁴. A region of 30 kilobases (kb) located at position 886–916 kb contains as much as 41.6% non-coding DNA and 11.5% pseudogenes. The non-coding DNA in this region has a small, but significantly higher, G+C content (mean 27.3%) than non-coding DNA in other areas of the genome (mean 23.7%) ($P < 0.001$), indicating that it may correspond to inactivated genes that are being degraded by mutation (Fig. 3).

Origin of replication. The origin of replication has not been experimentally identified in the *R. prowazekii* genome, but we identified *dnaA* at ~750 kb. However, the genes flanking the *dnaA* gene differ from the conserved motifs found in *Escherichia coli* and *Bacillus subtilis* (*rnpA*–*rpmH*–*dnaA*–*dnaN*–*recF*–*gyrB*). In *R. prowazekii*, the genes *rnpA* and *rpmH* are located in the vicinity of *dnaA*, but in the reverse orientation compared to the consensus motif, and *dnaN*, *recF* and *gyrB* are located elsewhere.

The origin and end replication in microbial genomes are often associated with transitions in GC skew ($G - C/G + C$) values²⁴. In *R. prowazekii* we observe transitions in the GC skew values at

around 0 and 500–600 kb (Fig. 1). There is a weak asymmetry in the distribution of genes in the two strands, such that the first half of the genome has a 1.6-fold higher gene density on one strand and the second half of the genome has a 1.6-fold higher gene density on the other strand. The shift in coding-strand bias correlates with the shift in GC-skew values. As most genes are transcribed in the direction of replication in microbial genomes, the origin of replication may correspond to the shift in GC-skew values at the position that we have chosen as the start point for numbering. Indeed, several short sequence stretches that are characteristic of *dnaA*-binding motifs are found in the intergenic region of genes *RP001* and *RP885* at 0 kb, supporting this interpretation.

Stable RNA sequences and repeat elements. We identified 33 genes encoding transfer RNA, corresponding to 32 different isoacceptor-tRNA species. There is a single copy of each of the rRNA genes, with *rrs* located more than 500 kb away from the *rrl*–*rrf* gene cluster around 0 and 500–600 kb (Fig. 1). There is a weak asymmetry in the distribution of genes in the two strands, such that the first half of the genome has a 1.6-fold higher gene density on one strand and the second half of the genome has a 1.6-fold higher gene density on the other strand. The shift in coding-strand bias correlates with the shift in GC-skew values. As most genes are transcribed in the direction of replication in microbial genomes, the origin of replication may correspond to the shift in GC-skew values at the position that we have chosen as the start point for numbering. Indeed, several short sequence stretches that are characteristic of *dnaA*-binding motifs are found in the intergenic region of genes *RP001* and *RP885* at 0 kb, supporting this interpretation.

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Figure 2 Linear map of the *R. prowazekii* chromosome. The position and orientation of known genes are indicated by arrows. Coding regions are colour-coded according to their functional roles. The positions of tRNA genes are indicated (inverted triangle on stalk). For additional information, see <http://evolution.bmc.uu.se/~siv/gnomics/Rickettsia.html>.

(Fig. 1). Comparison of the sequences from ten different *Rickettsia* species indicates that the disruption of the rRNA gene operon preceded the divergence of the typhus group and spotted fever group *Rickettsia* (S.G.E.A. *et al.*, unpublished observations). In addition, the genome contains a short sequence with similarity to a 213-nucleotide RNA molecule in *Bradyrhizobium japonicum* that may regulate transcription²⁵.

There are unusually few repeat sequences in this genome. We identified four different types of repeat sequence: all of these are located in intergenic regions. There is a sequence of 80 bp that is repeated seven times downstream of *rpmH* and *rnpA* in the *dnaA* region. A repetitive sequence of 325 bp is found at two intergenic regions that are more than 80 kb apart, downstream of the genes *ksgA* and *rnh*, respectively. A 440-bp-long repetitive sequence has been identified at two intergenic sites, 140 kb apart; one of these sites is downstream of *rff* and the others downstream of *pdhA* and

pdhB. Finally, two similar sequences of 730 bp are located immediately next to each other at 850 kb.

Paralogous families. We have identified 54 paralogous gene families comprising 147 gene products. Of these, 125 have an assigned function. Most paralogues encode proteins with transport functions, such as the ABC transporters, the proline/betaine transporters and the ATP/ADP transporters. Five paralogous genes located next to each other at 115 kb encode putative integral membrane proteins with unknown functions.

Biosynthetic pathways

A striking feature of the *R. prowazekii* genome is the small proportion of biosynthetic genes compared with free-living proteobacterial relatives (such as *Haemophilus influenzae*, *Helicobacter pylori* and *E. coli*)^{15,19,20}. This scarcity of biosynthetic functions is also seen in diverse endocellular and epicellular parasites^{16–18,23}. This scarcity of biosynthetic functions is also seen in diverse endocellular and epicellular parasites^{16–18,23}.

Amino-acid metabolism. As many as 43 and 69 genes required for amino-acid biosynthesis are found in *Helicobacter pylori* and *Haemophilus influenzae*, respectively. In contrast, *Mycoplasma genitalium* and *Borrelia burgdorferi* contain only *glyA*, which encodes serine hydroxymethyltransferase. This gene is also found in *R. prowazekii* (Table 1). Serine hydroxymethyltransferase catalyses the conversion of serine and tetrahydrofolate into glycine and methylenetetrahydrofolate, respectively. A role in tetrahydrofolate metabolism may account for the ubiquity of *glyA* in bacteria.

Seven genes normally associated with lysine biosynthesis (*lysC*, *asd*, *dapA*, *dapB*, *dapD*, *dapE* and *dapF*) are also present in *R. prowazekii*. The biosynthetic pathways leading to lysine, methionine and threonine share the first two of these (*lysC* and *asd*). However, none of the downstream genes for threonine biosynthesis are found in *R. prowazekii*. Likewise, the lysine pathway is incomplete, and *lysA*, which encodes the enzyme that converts meso-diaminopimelate to lysine, is missing. The likely role of the upstream genes of this pathway in *R. prowazekii* is the biosynthesis of diaminopimelate, an essential envelope component. We have therefore classified these genes as 'cell-envelope' genes (Table 1).

We have identified other genes that are superficially involved in the metabolism of amino acids, but which apparently function in deamination pathways that divert amino acids into the tricarboxylic acid (TCA) cycle. For example, there is *aatA*, encoding aspartate aminotransferase, which catalyses the degradation of aspartate to oxaloacetate and glutamate. *tdcB* encodes threonine deaminase, which converts threonine into α -ketobutyrate. Another gene (*ilvE*) encodes branched-chain-amino-acid aminotransferase, which converts leucine, isoleucine or valine into glutamate. *pccA* and *pccB* encode propionyl-CoA carboxylase, which converts propionyl-CoA, an intermediate in the breakdown of methionine, valine and isoleucine, into succinyl-CoA. The *pccA* and *pccB* gene products show greatest similarity to the eukaryotic proteins that are located in the mitochondrial matrix.

Nucleotide biosynthesis. No genes required for the *de novo* syntheses of nucleosides have been found in the *R. prowazekii* genome. However, four genes required for the conversion of nucleoside monophosphates into nucleoside diphosphates (*adk*, *gmk*, *cmk* and *pyrH*) are present. There are also two genes encoding ribonucleotide reductase, which converts ribonucleoside diphosphates into deoxyribonucleoside diphosphates. Nucleoside diphosphate kinase (encoded by *ndk*), which converts NDPs and dNDPs to NTPs and dNTPs, is also present in *R. prowazekii*. Finally, there is a complete set of genes for the conversion of dCTP and dUTP into TTP, including *thyA*, which codes for thymidylate synthase. Thus, the *R. prowazekii* genome encodes all of the enzymes required for the interconversion of nucleoside monophosphates into all of the other required nucleotides. The nucleoside monophosphates are probably imported from the eukaryotic host.

Table 1 Asterisks indicate putative pseudogenes. Abbreviations of species names are: Bacteria: *Acinetobacter calcoaceticus* (B-Aca), *Actinobacillus actinomycetem-comitans* (B-Aac), *Acyrtosiphon condii* (B-Aco), *Agrobacterium tumefaciens* (B-Atu), *Alcaligenes eutrophus* (B-Aeu), *Anabena* sp. PCC7120 (B-Asp), *Anabena variabilis* (B-Ava), *Anacystis nidulans* (B-Ani), *Azorhizobium caulinodans* (B-Aca), *Azospirillum brasilense* (B-Abr), *Azotobacter vinelandii* (B-Avi), *Bacillus caldote-nax* (B-Bca), *Bacillus stercorophilus* (B-Bst), *Bacillus subtilis* (B-Bsu), *Bartonella bacilliformis* (B-Bba), *Bartonella henselae* (B-Bhe), *Bordetella pertussis* (B-Bpe), *Borrelia burgdorferi* (B-Bbu), *Bradyrhizobium japonicum* (B-Bja), *Brucella abortus* (B-Bab), *Brucella ovis* (B-Bov), *Caulobacter crescentus* (B-Ccr), *Chlamydia trachomatis* (B-Ctr), *Chloroflexus aurantiacus* (B-Cau), *Chromatium viosum* (B-Cvi), citrus-greening-disease-associated bacterium (B-Cgr), *Clostridium acetobutylicum* (B-Cac), *Clostridium pasteurianum* (B-Cpa), *Clostridium thermosaccharolyticum* (B-Cts), *Coxiella burnetii* (B-Cbu), *Erwinia chrysanthemi* (B-Ech), *Escherichia coli* (B-Eco), *Haemophilus influenzae* (B-Hin), *Helicobacter pylori* (B-Hpy), *Klebsiella pneumoniae* (B-Kpn), *Legionella pneumo-phil* (B-Lpn), *Leuconothrix mucor* (B-Lmu), *Liberobacter africanum* (B-Laf), *Methylobacterium extorquens* (B-Mex), *Micrococcus luteus* (Mlu), *Moraxella catarrhalis* (Mca), *Mycobacterium leprae* (Mle), *Mycobacterium smegmatis* (B-Msm), *Mycobacterium tuberculosis* (B-Mtu), *Mycoplasma capricolum* (B-Mca), *Mycoplasma genitalium* (B-Mge), *Mycoplasma pneumoniae* (B-Mpn), *Paracoccus denitrificans* (B-Pde), *Pasteurella haemolytica* (B-Pha), *Plectonema boryanum* (B-Pbo), *Proteus mirabilis* (B-Pmi), *Proteus vulgaris* (B-Pvu), *Pseudomonas aeruginosa* (B-Pae), *Pseudomonas fluorescens* (B-Pfl), *Pseudomonas putida* (B-Ppu), *Pseudomonas syringae* (B-Psy), *Rhizobium meliloti* (B-Rme), *Rhizobium* sp. NGR234 (B-Rsp), *Rhodobacter capsulatus* (B-Rca), *Rhodobacter sphaeroides* (B-Rsp), *Rhodobacter sulfidophilus* (B-Rsu), *Rhodospseudomonas blastica* (B-Rbl), *Rhodospirillum rubrum* (B-Rru), *Rickettsia japonicum* (B-Rja), *Rickettsia rickettsii* (B-Rri), *Rickettsia typhi* (B-Rty), *Salmonella typhi* (B-Sti), *Salmonella typhimurium* (B-Sty), *Shigella flexneri* (B-Sfl), *Spirioplasm citri* (B-Sci), *Staphylococcus aureus* (B-Sau), *Staphylococcus carnosus* (B-Scs), *Streptococcus pneumoniae* (B-Spn), *Streptomyces clavuligerus* (B-Sol), *Streptomyces coelicolor* (B-Scs), *Synechocystis* PCC 6803 (B-Syn), *Thermus aquaticus* (B-Taq), *Thermus thermophilus* (B-Tth), *Thiobacillus cuprinus* (B-Tcu), *Treponema hyodysenteriae* (B-Thy), *Vibrio alginolyticus* (B-Val), *Vibrio cholera* (B-Vch), *Vibrio parahaemolyticus* (B-Vpa), *Vibrio proteolyticus* (B-Vpr), *Wolbachia* sp. (B-Wsp), *Yersinia enterocolitica* (B-Yen), *Zooglea ramigera* (B-Zra), *Zymomonas mobilis* (B-Zmo). Archaea: *Methanococcus jannaschii* (A-Mja), *Sulfolobus acidocaldarius* (A-Sac). Eukaryotes: *Apis mellifera* (E-Ame), *Arabidopsis thaliana* (E-Ath), *Atratyloides japonica* (E-Aja), *Bos taurus* (E-Bta), *Candida albicans* (E-Cal), *Caenorhabditis elegans* (E-Cel), *Dictyostellium discoideum* (E-Ddi), *Flaveria trinervia* (E-Ftr), *Giardia theta* (E-Gth), *Glycine max* (E-Gma), *Haematobia irritans* (E-Hir), *Homo sapiens* (E-Hsa), *Marchantia polymorpha* (E-Mpa), *Mus musculus* (E-Mmu), *Prototheca wickerhamii* (E-Pwi), *Petunia hybrida* (E-Phy), *Pisum sativum* (E-Psa), *Porphyra purpurea* (E-Ppu), *Odontella sinensis* (E-Osi), *Reclinomonas americana* (E-Ram), *Rattus norvegicus* (E-Rno), *Rhizopus oryzae* (E-Ror), *Saccharomyces cerevisiae* (E-Sce), *Schizosaccharomyces pombe* (E-Spo), *Solanum tuberosum* (E-Stu), *Spinacia oleracea* (E-Sol).

Energy metabolism

Early in its infectious cycle, *R. prowazekii* uses the ATP of the host with the help of membrane-bound ATP/ADP translocases. However, *R. prowazekii* is also capable of generating ATP, which may compensate for the gradual depletion of cytosolic ATP later in the infection. *R. prowazekii*'s repertoire of genes involved in ATP production and transport include determinants for the TCA cycle, the respiratory-chain complexes, the ATP-synthase complexes and the ATP/ADP translocases (Table 1). Genes to support anaerobic glycolysis are absent.

Pyruvate dehydrogenase. Pyruvate is imported into mitochondria directly from the cytoplasm and converted into acetyl-CoA by pyruvate dehydrogenase. The genes encoding three components (E1–E3) of the pyruvate dehydrogenase complex are found in *R. prowazekii*, indicating that it too uses cytosolic pyruvate. Pyruvate dehydrogenase (E1) consists of two subunits (α and β) in *R. prowazekii*, mitochondria and Gram-positive bacteria; the corresponding genes are clustered in the genome. In contrast, proteobacteria such as *E. coli*, *Haemophilus influenzae* and *Helicobacter pylori* have a single subunit for the E1 component and these have little similarity to the α and β subunits of the E1 component in *R. prowazekii* and mitochondria (data not shown).

Two paralogous genes code for the dihydrolipoamide dehydrogenase (E3) in *R. prowazekii*. One of these most resembles mitochondrial homologues, whereas the other is most similar to bacterial homologues (data not shown). The presence of several paralogous gene families for pyruvate dehydrogenases complicates attempts to reconstruct a genome phylogeny based on these genes.

ATP production. Genes encoding all enzymes in the TCA cycle are found in *R. prowazekii*. Proton translocation is mediated by NADH dehydrogenase (complex I), cytochrome reductase (complex III) and cytochrome oxidase (complex IV). Several clusters of genes code for components of the NADH dehydrogenase complex. Seven of these genes (*nuoJKLM* and *nuoGHO*) are located near to each other, but the order of genes is inverted relative to the order of this cluster in *E. coli*. An additional set of five genes is grouped in the order *nuoABCDE*, but the single genes *nuoF* and *nuoN* are distant from both of these clusters. Several proteins in the cytochrome *bc*₁ reductase complex, such as ubiquinol–cytochrome *c* reductase

(encoded by *petA*), cytochrome *b* (encoded by *cytb*) and cytochrome *c*₁ (encoded by *fbhC*), are present, as are several subunits of the cytochrome oxidase complex.

The ATP-synthesizing complex is composed of the ATP synthase F₁ component (comprising five polypeptides, α , β , γ , ϵ and δ) and the F₀ component, a hydrophobic segment that spans the inner mitochondrial membrane. The genes encoding these components are normally clustered in one of the most highly conserved operon structures in microbial genomes. In *R. prowazekii*, however, the ATP-synthase genes encoding the α , β , γ , δ and ϵ subunits of the F₁ complex (*atpH*, *atpA*, *atpG*, *atpD* and *atpC*) are clustered in the common order, but *atpB*, *atpE* and *atpF*, encoding the A, B and C chains of the F₀ complex, are split from this cluster.

Replication, repair and recombination

R. prowazekii has a smaller set of genes involved in DNA replication than do free-living bacteria such as *E. coli*, *Haemophilus influenzae* and *Helicobacter pylori*. Four genes have been identified that code for the core structure of DNA polymerase III, which includes the α (*dnaE*), ϵ (*dnaQ*), β (*dnaN*), γ and θ (*dnaX*) subunits. Extra subunits present in the *E. coli* DNA polymerase III are missing from *R. prowazekii*, as well as from *M. genitalium* and *B. burgdorferi*.

Genes encoding DNA-repair mechanisms are similar in the small genomes of the parasites *R. prowazekii*, *M. genitalium* and *B. burgdorferi*. Thus, genes involved in the repair of ultraviolet-induced DNA damage (*uvrABCD*) have been identified in all three genomes. In *R. prowazekii*, DNA-excision repair probably occurs by a pathway involving endonuclease III, polI and DNA ligase, as in *B. burgdorferi*.

The *R. prowazekii* genome has a limited capacity for mismatch repair. The DNA-mismatch-repair enzymes encoded by *mutL* and *mutS* are present, but *mutH* and *mutY* are not. There is a complete lack of *mut* genes in *M. genitalium*, but *mutL* and *mutHLY* have been identified in *B. burgdorferi* and *Chlamydia trachomatis*. The transcription-repair coupling factor (encoded by *mfd*) is found in *R. prowazekii*, *B. burgdorferi* and *C. trachomatis* but not in *M. genitalium*.

The *R. prowazekii* genome contains several genes involved in homologous recombination, such as *recA*, *recF*, *recJ*, *recN* and *recR*. A similar set of genes has been found in *A. aeolicus*²¹. The *rec* genes

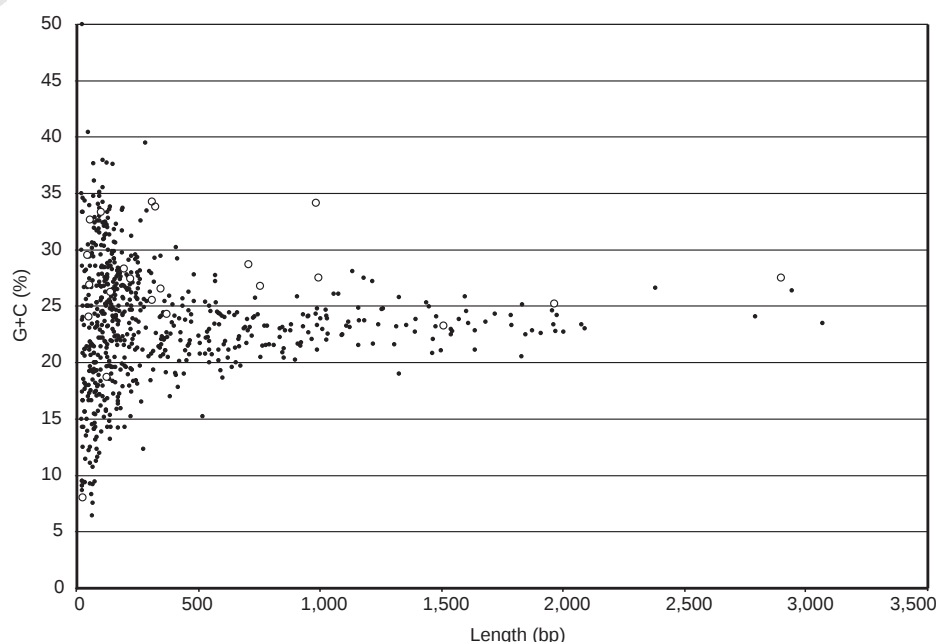


Figure 3 G+C content in intergenic regions longer than 20 bp in the *R. prowazekii* genome. The empty circles correspond to spacer sequences located at 886 to

916 kb, a region with an unusually large fraction of non-coding DNA and pseudogenes.

are scattered in the other small genomes of parasites. The *RecBCD* complex is missing in *R. prowazekii*, *M. genitalium* and *Helicobacter pylori* but it has been identified in *B. burgdorferi*.

Transcription and translation

R. prowazekii has three subunits (α , β and β') of the core RNA polymerase, as well as σ^{70} and one alternative σ factor, σ^{32} , which controls transcription of the genes encoding heat-shock proteins in *E. coli*. Genes involved in transcription elongation and termination, *nusA*, *nusB*, *nusG*, *greA* and *rho*, are also present. The gene encoding σ^{32} is absent in most other small genomes, such as those of *B. burgdorferi*, *Helicobacter pylori*, *M. genitalium* and *C. trachomatis*, although genes for heat-shock proteins are present.

An unusually large number of genes involved in RNA degradation are found in *R. prowazekii*. Of these, only four appear to be common to the bacterial genomes analysed so far (those encoding polynucleotide nucleotidyltransferase and ribonucleases HII, III and P). Four more ribonucleases (D, E, HI and PH) are present in *R. prowazekii*, but in none of the other small parasites.

Of the 33 identified tRNA genes, which code for 32 different tRNA isoacceptor species, two code for tRNA^{Phe}. There are two tRNA species for most of the amino acids that are encoded by four-codon boxes; the exceptions are the four-codon boxes for proline and valine, for which we have identified only one isoacceptor-tRNA species, with U in the first anticodon position. *selC*, which codes for tRNA^{Sec}, and *selABD* are missing. *R. prowazekii* has a set of genes coding for tRNA modifications (*tgt*, *queA*, *trmD*, *truA*, *truB* and *miaA*) which resembles that of *Helicobacter pylori*, *C. trachomatis* and *B. burgdorferi*; *M. genitalium* has only *trmD* and *truA*.

In *R. prowazekii*, 21 genes encode 18 of the 20 aminoacyl-tRNA synthetases normally required for protein synthesis. There are two genes (*gltX*) encoding glutamyl-tRNA synthetase. As seen in several bacterial genomes²⁵, the gene coding for glutaminyl-tRNA synthetase, *glnS*, is missing. Three genes encoding subunits of the glutamyl-tRNA amidotransferase are present, indicating that a glutamyl-tRNA charged with glutamic acid may be transamidated to generate Gln-tRNA. The gene coding for asparaginyl-tRNA synthetase, *asnS*, is also missing from the *R. prowazekii* genome as well as from *Helicobacter pylori*, *C. trachomatis* and *A. aeolicus*²⁶. A transamidation process to form Asn-tRNA^{Asn} from Asp-tRNA^{Asn} has been proposed for the archaeon *Haloferax volcanii*²⁷ and this reaction may also occur in *R. prowazekii*. The valyl-tRNA synthetase is 38.3% identical to its homologue in *Methanococcus jannaschii*, but only 27.6% identical to its most similar homologue in bacteria, which is found in *Bacillus stearothermophilus*, possibly indicating a horizontal transfer event. The lysyl-tRNA synthetase (encoded by *lysS*) in *R. prowazekii* is a class I enzyme with no resemblance to the conventional class II lysyl-tRNA synthetases. Class I type of lysyl-tRNA synthetases have been observed previously in only *B. burgdorferi*, *Pyrococcus woesei*, *Methanococcus jannaschii* and a few other methanogens²⁶.

Regulatory systems

As in other genomes of small parasites, *R. prowazekii* has a reduced set of regulatory genes. There are a few members of two-component regulatory systems, such as the proteins encoded by *barA*, *envZ*, *ntrY*, *ntrX*, *ompR* and *phoR*. *spoT*, which is involved in the stringent response, has been identified in *B. burgdorferi*, *Helicobacter pylori* and *M. genitalium*. Only remnants of genes coding for amino-terminal fragments of proteins similar to those encoded by *spoT* and *relA* are identifiable in *R. prowazekii*. No fragments of *spoT* encoding the carboxy-terminal segments of the protein have been identified in the genome.

Cell division and protein secretion

Proteins involved in detoxification, such as superoxide dismutase, and those involved in thiophen and furan oxidation are present in *R.*

prowazekii. Two genes encoding haemolysins have also been identified, and an *R. typhi* homologue of *tlyC* exhibits haemolytic activities when expressed in *E. coli* (S. Radulovic, J. M. Troyer, B. Noden, S.G.E.A. and A. Azad, unpublished observations).

The data indicate that the basic mechanisms of cell division and secretion in *R. prowazekii* are similar to those in free-living proteobacteria. There is a common set of bacterial chaperones (encoded by *dnaK*, *dnaJ*, *hslU*, *hslV*, *groEL*, *groEL*, *groES* and *hspG*) and genes involved in the *secA*-dependent secretory system (*secABDEFGY*, *ffh* and *ftsY*). *R. prowazekii* has a significantly larger set of genes involved in peptide secretion than does *M. genitalium*.

Membrane-protein analysis

Many studies of *R. prowazekii* have focused on outer-surface membrane proteins because of their potential importance in bacterial detection and vaccination. The superficial lipopolysaccharide (LPS) molecule is important in the pathogenesis of *R. prowazekii*. LPS consists of a polysaccharide that is covalently linked to lipid A, the biosynthesis of which is catalysed by products of *lpxABCD*, all of which are present in the *R. prowazekii* genome. These genes are clustered in *E. coli*, but *lpxA* and *lpxD* are separate from *lpxB* and *lpxC* in *R. prowazekii*. Three genes involved in the biosynthesis of the 3-deoxy-D-manno-octulosonic acid (KDO) residues reside in the *R. prowazekii* genome (*kdsA*, *kdsB* and *kdtA*). Only one gene (*rfaJ*) with a putative function in outer-core biosynthesis has been identified.

We have identified a set of genes involved in the biosynthesis of murein and diaminopimelate and a set involved in the biosynthesis of fatty acids. These includes: *fabD*, which is involved in the last step of the initiation phase of fatty-acid biosynthesis; four genes involved in the elongation cycle of fatty-acid biosynthesis (*fabFGHI*); and three genes involved in the first three steps of the synthesis of polar head groups (*cdsA*, *pssA* and *pgsA*). Finally, post-translational processing and addition of lipids to an N-terminal cysteine require the gene products prolipoprotein diacylglycerol transferase (*lgt*), prolipoprotein signal peptidase (*lspA*) and apolipoprotein:phospholipid N-acyl transferase (*lnt*). These are found in the genome with several genes involved in the degradation of fatty acids, such as *fadA* which encodes the 3-ketoacyl-CoA thiolase.

Virulence

The *R. prowazekii* genome contains several homologues of the *VirB* gene operon found in *Agrobacterium tumefaciens*. This gene family encodes proteins that direct the export of the T-DNA-protein complex across the bacterial envelope to the plant nuclei²⁸. *R. prowazekii* has two homologues of *VirB4* and one homologue each of *VirB8*, *VirB9*, *VirB10*, *VirB11* and *VirD4*. The latter five genes are clustered with the gene *trbG*, which is involved in conjugation in *Agrobacterium tumefaciens*. Homologues of the single-stranded DNA-binding proteins *VirD2* and *VirE2* are missing. In *Agrobacterium tumefaciens*, these proteins are bound to the transferred T-DNA, indicating different functions for the homologues of the *VirB* genes in *R. prowazekii*. Indeed, *VirB* proteins are homologous to components of the *E. coli* transport system for plasmids, as well as to components of the Pt1 transport machinery in *Bordetella pertussis*, which exports pertussis toxin²⁸. A set of genes coding for *VirB4* and several other *VirB* proteins has been identified in the *cag* pathogenicity island of *Helicobacter pylori*. In this species, the *VirB* proteins facilitate export of a factor that induces interleukin-8 secretion in gastric epithelial cells²⁸. Thus, *R. prowazekii* may encode components of a transport system for both conjugal DNA transfer and protein export.

The virulence of *Staphylococcus aureus* has been correlated with the production of capsular polysaccharides in phagocytic assays and mouse lethality assays^{29,30}. A cluster of ten capsule genes (*capA-M*) is involved in capsule biosynthesis in *S. aureus* strain M³¹. We have identified three *R. prowazekii* genes with sequence similarities to *S. aureus cap* genes. Two of these (*capD* and *capM*) are separated by ten

genes, most of which are unknown genes or genes involved in the biosynthesis of LPS or teichoic acid. Thus, *R. prowazekii* may produce components of a microcapsular layer that is involved in virulence.

Reductive evolution

Genome sequences of organisms enjoying an endosymbiotic life-style are at risk. The activities of homologous nuclear genes may render genes of the endosymbiont expendable and as a consequence they become vulnerable to obliteration by mutation. Good candidates for such purged genes in *Rickettsia* and mitochondria are genes required for amino-acid biosynthesis, nucleoside biosynthesis and anaerobic glycolysis. These and other genes would have been deleted when an ancestral genome first lived in a nucleated cell. Once genes essential to a free-living mode are lost, the endosymbiont becomes an obligate resident of its host.

Likewise, small, bottle-necked populations of bacteria infecting a eukaryotic cell will tend to accumulate deleterious mutations because selection cannot remove them from such clonal populations¹³. The accumulation of such harmful but non-lethal mutations is referred to as 'Muller's ratchet'³² or 'near-neutral evolution'^{33,34}. The consequence of accumulation of these mutations will be the inactivation and eventual deletion of non-essential genes.

The first mutation that inactivates an expendable gene is likely to initiate a sequence of events in which subsequent mutations freely transform it, by degrees, from a pseudogene, to unrecognizable sequence, to small fragments, to extinction. In this sequence, mutations are released from amino-acid-coding constraints. Thus nucleotide substitutions will reflect the mutation bias of the genome. This bias can be estimated roughly by frequencies of third-position bases in the codons. For *R. prowazekii*, the bias of the third-position bases is 18% G+C rather than the 29% G+C average for the genome. So, as sequences age in *R. prowazekii*, their composition should gradually approach the low G+C content of third codon positions. Nearly one-quarter of the *R. prowazekii* genome is composed of non-coding sequences, with a G+C content lower than that of coding sequences (25% G+C compared to 30%; $P < 0.001$). Thus, much of the non-coding sequence may be remnants of coding sequences that are in the process of being eliminated from the genome.

The gene encoding S-adenosylmethionine synthetase (*metK*), which catalyses the biosynthesis of S-adenosylmethionine (SAM), illustrates the initiation of this process. The *metK* sequence in the strain of *R. prowazekii* studied here has a termination codon within a region of the gene that is otherwise highly conserved among

bacterial species³⁵. However, a closely related strain does not have the termination codon. Many other defects, such as termination codons, insertions, and a preponderance of small deletions, have also been observed in the *metK* genes in several members of the spotted fever group *Rickettsia* (J.O.A. and S.G.E.A., unpublished observations). This random distribution of lethal mutations among some *metK* alleles from different *Rickettsia* species indicates that the gene may have just entered the extinction process. This distribution, and the identification of 11 more pseudogenes for carboxypeptidase (*ypwA*), penicillin-binding protein (*pbpC*), succinyl CoA-transferase (*scoB*), transposase (*tra3*), resolvase (*pin*), conjugative transfer protein (*taxB*), a hypothetical protein (*yfc1*) and four different fragmented open reading frames for (p)ppGpp 3'-pyrophosphohydrolase, indicates that the *R. prowazekii* genome continues to eliminate genes.

Genome sequences can be purged by a more abrupt mechanism. This consists of intrachromosomal recombination at duplicated sequences, which can result in the deletion of intervening sequences, the loss of a sequence duplication and the rearrangement of flanking

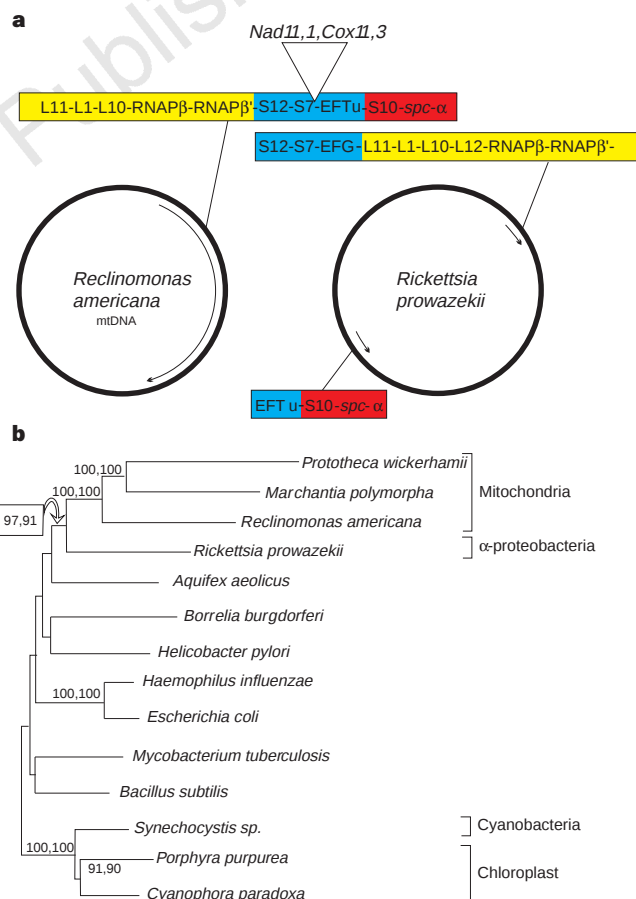


Figure 5 The organization and phylogenetic relationships of gene encoding ribosomal protein from *R. prowazekii* and the mitochondrial genome of *Reclinomonas americana*. **a**, The organization of ribosomal-protein genes. The S10, *spc* and α -operons are organized similarly in these two genomes, except that several ribosomal-protein genes³⁸ have been deleted from the mitochondrial genome of *Reclinomonas americana*. **b**, The phylogenetic relationships of mitochondria and bacteria were derived from the combined amino-acid sequences of ribosomal proteins S2, S3, S7, S10, S11, S12, S13, S14, S19, L5, L6 and L16. Neighbour-joining and maximum-parsimony methods gave identical topologies. Branch lengths are proportional to those reconstructed by using the neighbour-joining method. Values at nodes are bootstrap values indicating the degree of support for individual clusters under each method (neighbour-joining, maximum parsimony). Only bootstrap values >90% are shown.

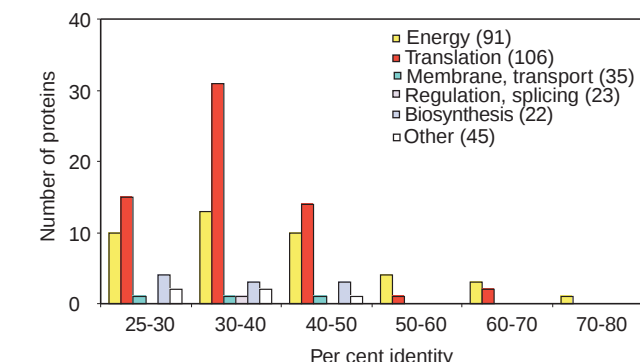


Figure 4 Histogram representation of the similarity of predicted *R. prowazekii* proteins to yeast proteins targeted to the mitochondria. Only protein pairs with per cent identity values greater than 25% are shown. Numbers in parentheses represent the total number of yeast mitochondrial proteins within each category. The yeast mitochondrial protein sequences have been taken from <http://www.proteome.com>.

sequences. Such a mechanism will account for the presence in *R. prowazekii* of one, unlinked copy of *rrs* and *rrl*, both of which are surrounded by new flanking sequences³⁶. Likewise, *R. prowazekii* has one *tuf* gene and one *fus* gene in atypical clusters that seem to have been created by intrachromosomal recombination between the two *tuf* genes that are normally found in Gram-negative bacteria³⁷. Indeed, rearranged gene operon structures encoding ribosomal proteins are characteristic of all members of the genus *Rickettsia* (H. Amiri, C.A. and S.G.E.A., unpublished observations).

Conserved operons that are found in free-living bacteria are often dispersed throughout the *Rickettsia* genome (see above). The *R. prowazekii* genome contains an unusually small fraction of repeat sequences (<10% of that observed in free-living bacteria). We suggest that the repeat sequences found in the ancestor to the *Rickettsia* have been 'consumed' by the intrachromosomal-recombination mechanism that generated some of the deletions and rearrangements seen in *R. prowazekii*. Such intrachromosomal recombinants arise at a substantial rate in bacteria growing in culture, but here they are eliminated from the populations by selection. That such remnants of intrachromosomal recombination are retained in *R. prowazekii* indicates that purifying selection has been attenuated in this organism.

Mitochondrial affinities

The reduction in genome size in mitochondria and *Rickettsia* is likely to have occurred independently in the two lineages. Most of

the genes supporting mitochondrial activities are nuclear. Many of the 300 proteins encoded in the nucleus of the yeast *Saccharomyces cerevisiae* but destined for service within the mitochondrion are close homologues of their counterparts in *R. prowazekii*. Nearly one-quarter of these proteins are required for bioenergetic processes and another one-third of them are required for the expression of the genes encoded in the mitochondrial genome. In total, more than 150 nucleus-encoded mitochondrial proteins share significant sequence homology with *R. prowazekii* proteins (Fig. 4).

Another group of 58 nucleus-encoded mitochondrial proteins represents components of the mitochondrial transport machinery and regulatory system (Fig. 4). These include proteins found in the mitochondrial outer membrane and others involved in splicing reactions. Such proteins have probably been secondarily recruited to mitochondria from genomes not necessarily related to that of the α -proteobacterial ancestor.

The mitochondrial genome of the early diverging, freshwater protozoan *Reclinomonas americana* is more like that of a bacterium than any other mitochondrial genome sequenced so far³⁸. This genome contains 67 protein-coding genes, most of which provide components of genetic processes and the bioenergetic system³⁸. Several gene clusters in this mitochondrial genome are reminiscent of those in bacteria (Figs 5a, 6a). Most similarities represent retained, ancestral traits present in the common ancestor of bacteria and mitochondria. For example, the genes *rplKAJL* and *rpoBC* are identically organized in *R. prowazekii* and the mitochondrial genome of *Reclinomonas americana*. Likewise, the genes encoding the S10, *spc* and the α -ribosomal protein operons are organized similarly in the two genomes. The immediate proximity of these two clusters in the *Reclinomonas americana* mitochondrial DNA is reminiscent of the arrangement in free-living bacteria, whereas the physical separation of the two clusters in the *R. prowazekii* genome is atypical. A further rearrangement event is indicated by the fact that the *rpsLrpsGfus* cluster is located upstream of the *rplKAJLrpoBC* cluster in *R. prowazekii*, rather than downstream as it is in the *Reclinomonas americana* mtDNA. Phylogenetic reconstructions based on ribosomal proteins within each of these two clusters indicate that there is a close evolutionary relationship between *R. prowazekii* and mitochondria (Fig. 5b).

Mitochondria and *R. prowazekii* have a similar repertoire of proteins involved in ATP production and transport, including genes encoding components of the TCA cycle, the respiratory-chain complexes, the ATP-synthase complexes and the ATP/ADP translocases. There are some similarities in the gene orders of some functional clusters (Fig. 6a). There are also some rearrangements of clusters that are specific to *Rickettsia*. One example is the inversion of segments corresponding to *nuoJKLM* and *nuoGHI*. Another is the scattered displacement of genes involved in the biogenesis of cytochrome *c*. Nevertheless, phylogenetic reconstructions based on components of the NADH dehydrogenase complexes indicate that there is a close evolutionary relationship between *R. prowazekii* and mitochondria (Fig. 6b).

We have identified as many as five genes coding for ATP/ADP transporters, all of which are expressed (R.M.P. *et al.*, unpublished observations). The *Rickettsia* ATP/ADP translocases are monomers with 12 transmembrane regions each, whereas the mitochondrial translocases are dimers with six transmembrane regions per dimer. We found no relationship between the primary structures of the mitochondrial and *Rickettsia* ATP/ADP translocases, indicating that these transport systems may have originated independently.

The study of the *R. prowazekii* genome sequence supports the idea that aerobic respiration in eukaryotes originated from an ancestor of the *Rickettsia*, as indicated previously by phylogenetic reconstructions based on the rRNA gene sequences^{7,9}. Phylogenetic analyses of the *petB* and *coxA* genes indicate that the respiration systems of *Rickettsia* and mitochondria diverged ~1,500–2,000 million years ago¹⁰, shortly after the amount of oxygen in the atmosphere began

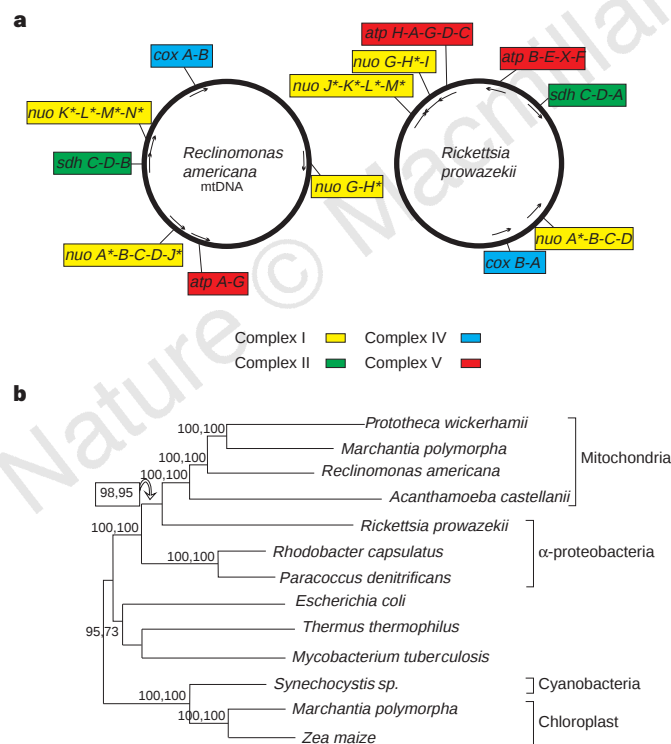


Figure 6 The organization and phylogenetic relationships of genes involved in ATP synthesis from *R. prowazekii* and the mitochondrial genome of *Reclinomonas americana*. **a**, The organization of bioenergetic genes. **b**, The phylogenetic relationships of mitochondria and bacteria were derived from the combined amino-acid sequences of NADH dehydrogenase I chains A, J, K, L, M and N, which are encoded by the genes *nuoA*, *J*, *K*, *L*, *M*, *N*. These genes are highlighted by asterisks in **a**. Neighbour-joining and maximum-parsimony methods gave identical topologies. Branch lengths are proportional to those reconstructed using the neighbour-joining method. Values at nodes are bootstrap values indicating the degree of support for individual clusters under each method (neighbour-joining, maximum parsimony). Only bootstrap values >90% are shown.

to increase. The finding that the ATP/ADP translocases in *R. prowazekii* and mitochondria are of different evolutionary origin is problematic (R.M.P. *et al.*, unpublished observations). Free-living bacteria do not seem to have homologues of ATP/ADP translocases, which are found only in organelles and in two obligate intracellular parasites, *Rickettsia* and *Chlamydia*. Thus it is not known whether the original endosymbiont was capable of efficient exchange of adenosine nucleotides with its host cell. More detailed comparative analysis of the genomes of α -proteobacteria may refine our understanding of the origins of mitochondria. □

Methods

Genome sequencing. We prepared genomic DNA from the Madrid E strain of *R. prowazekii*, which was originally isolated in Madrid from a patient who died in 1941 with epidemic typhus. We propagated *R. prowazekii* in the yolk sac of embryonated hen eggs and purified DNA according to standard procedures³⁹. We sequenced the *R. prowazekii* genome by a whole-genome shotgun approach in combination with shotgun sequencing of a selected set of clones from a cosmid library (A.Z. *et al.*, unpublished observations). Genomic and cosmid DNA was sheared by nebulization to an average size of ~2 kb. The random fragments were cloned into a modified M13 vector using the double adaptor method⁴⁰. We collected 19,078 sequence reads during the random sequencing phase using Applied Biosystems 377 DNA sequencers (Perkin-Elmer).

The sequences were assembled and the consensus sequence was edited using the STADEN program⁴¹. We verified the structure of the assembled sequence by end-sequencing of 3-kb-insert λ Zap II clones³⁶, 10-kb λ clones and 30-kb cosmid clones. More than 97% of the genome was covered by clones from the three different libraries (A.Z. *et al.*, unpublished observations). Gaps between contigs were closed by direct sequencing of clones from the three libraries or of polymerase chain reaction (PCR) products. The final four gaps were closed by direct sequencing of PCR products generated with the Long Range PCR system (Gene Amp). Regions of ambiguity were identified by visual inspection of the assembly and resequenced. The final assembly contains ~20,000 sequences. The genome sequence has eightfold coverage on average and no single region has less than twofold coverage. We estimate the overall error frequency to be $< 1 \times 10^{-5}$.

Informatics. Sequence analysis and annotation was managed by CapDB (T.S.-P. *et al.*, unpublished observations). We identified open reading frames of more than 50 codons as genes on the basis of their characteristic patterns in nucleotide-frequency statistics¹⁴ using BioWish⁴². The identified genes were analysed using the program BLASTX⁴³ to search for sequence similarities in EMBL, TrEMBL, SwissProt and in-house databases. We identified tRNA genes with the program tRNA scan-SE⁴⁴. Remaining frameshifts were considered to be authentic and annotated as pseudogenes. Families of paralogues were constructed using BLAST to search for sequence similarities within the *R. prowazekii* genome. Multiple alignments and phylogenetic trees for genes with significant sequence similarities to genes in the public databases were constructed automatically using CLUSTAL-W⁴⁵, Phylo_win⁴⁶ and GRS⁴⁷. The final annotation was based on manual inspection of the phylogenetic placement of *R. prowazekii* in the resulting gene trees.

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Correspondence and requests for materials should be addressed to C.G.K. (e-mail: chuck@xray.bmc.uu.se).



Table 1. Functional classification of *Rickettsia prowazekii* protein-coding genes. Gene numbers correspond to those in Fig. 2. Percentages represent per cent identities.

AMINO ACID METABOLISM 6		
<i>Amino acid biosynthesis</i>		
RP743 <i>glyA</i>	serine hydroxymethyl transferase (B-Mex, 60.9%)	
RP428 <i>ilvE</i>	branched-chain amino acid aminotransferase (B-Eco, 36.8%)	
<i>Amino acid degradation</i>		
RP091 <i>aatA</i>	aspartate aminotransferase (B-Rme, 55.6%)	
RP618 <i>pccA</i>	propionyl-CoA carboxylase α chain (E-Rno, 45.0%)	
RP619 <i>pccB</i>	propionyl-CoA carboxylase β chain (E-Hsa, 63.3%)	
RP449 <i>tdcB</i>	threonine dehydratase (E-Yeast, 35.3%)	
BIOSYNTHESIS OF COFACTORS 25		
<i>Folic acid</i>		
RP536 <i>folC</i>	folypolyglutamate dehydrogenase (B-Bsu, 34.5%)	
RP515 <i>folD</i>	methylene tetrahydrofolate dehydrogenase (Bsu, 46.3%)	
RP383 <i>folE</i>	GTP cyclohydrolase I (B-Syn, 48.1%)	
<i>Haem and porphyrins</i>		
RP841 <i>hemA</i>	delta-aminolevulinic synthase (B-Bja, 49.1%)	
RP539 <i>hemB</i>	delta-aminolevulinic dehydratase (B-Bja, 53.3%)	
RP466 <i>hemC</i>	prophobilinogen deaminase (B-Pmi, 39.1%)	
RP885 <i>hemE</i>	uroporphyrinogen decarboxylase (B-Rca, 42.6%)	
RP882 <i>hemF</i>	coproporphyrinogen III oxidase (E-Gma, 43.0%)	
RP884 <i>hemH</i>	ferrochelatase (B-Syn, 40.4%)	
RP847 <i>hemK</i>	protoporphyrinogen oxidase (E-Eco, 44.3%)	
RP175 <i>hemN</i>	oxygen-independent coproporphyrinogen II (B-Bsu, 34.4%)	
<i>Lipote</i>		
RP742 <i>lipA</i>	lipic acid synthetase (B-Hin, 50.5%)	
RP876 <i>lipB</i>	lipic acid ligase (B-Mtu, 35.6%)	
<i>Menaquinone and ubiquinones</i>		
RP190 <i>coq7</i>	ubiquinone biosynthesis prt Coq7 (E-Rno, 36.9%)	
RP479 <i>ispB</i>	octaprenyl-diphosphate synthase (E-Eco, 36.5%)	
RP686 <i>ubiA</i>	4-hydroxyphenylacetate octaprenyltransferase (B-Eco, 36.1%)	
RP541 <i>ubiX</i>	3-octaprenyl-4-hydroxybenzoate carboxylase (B-Eco, 53.2%)	
RP680 <i>ubiE</i>	ubiquinone biosynthesis methyltransferase (B-Eco, 44.0%)	
RP622 <i>ubiG</i>	3-demethylubiquinone methyltransferase (B-Eco, 39.1%)	
<i>Thio- and glutaredoxin</i>		
RP204 <i>grxC1</i>	glutaredoxin 3 (B-Eco, 50.0%)	
RP745 <i>grxC2</i>	glutaredoxin 3 (B-Syn, 50.0%)	
RP327 <i>tdpX1</i>	thioredoxin-peroxidase (B-Hpy, 54.0%)	
RP002 <i>trxA</i>	thioredoxin (B-Ani, 52.8%)	
RP445 <i>trxB1</i>	thioredoxin reductase (B-Hin, 52.0%)	
RP514 <i>trxB2</i>	thioredoxin reductase (B-Cpa, 28.4%)	
CELL ENVELOPE 59		
<i>Diaminopimelate</i>		
RP316 <i>asd</i>	aspartate-semialdehyde dehydrogenase (B-Vch, 43.3%)	
RP429 <i>dapA</i>	dihydrodipicolinate synthase (A-Mja, 39.6%)	
RP148 <i>dapB</i>	dihydrodipicolinate reductase (B-Hpy, 37.7%)	
RP194 <i>dapD</i>	tetrahydrodipicolinate N-succinyltransferase (B-Eco, 57.9%)	
RP874 <i>dapE</i>	succinyl-diaminopimelate desuccinylase (B-Hin, 37.5%)	
RP415 <i>dapF</i>	diaminopimelate epimerase (B-Hin, 35.0%)	
RP753 <i>lysC</i>	aspartokinase (B-Bst, 37.3%)	
<i>Membranes and lipoproteins</i>		
RP347 <i>asmA</i>	outer membrane assembly protein (B-Eco, 19.3%)	
RP446 <i>igtD</i>	poliproprotein diacylglycerol transferase (B-Vch, 29.1%)	
RP366 <i>int</i>	apolipoprotein N-acyltransferase (B-Hin, 29.1%)	
RP300 <i>nlpD</i>	lipoprotein (B-Hin, 22.4%)	
RP390 <i>ripA</i>	rare lipoprotein A (B-Hin, 23.9%)	
RP224 <i>tolC</i>	outer membrane protein (B-Eco, 22.9%)	
RP048 <i>ylpC</i>	inner membrane protein, 60 kDa (B-Hin, 30.4%)	
<i>Murein sacculus</i>		
RP095 <i>air</i>	alanine racemase (B-Hin, 29.5%)	
RP389 <i>dacF</i>	penicillin binding protein precursor (B-Bsu, 33.8%)	
RP249 <i>ddlB</i>	D-alanine-D-alanine ligase (B-Hin, 32.8%)	
RP454 <i>glmU</i>	UDP-N-acetylglucosamine pyrophosphorylase (B-Hin, 34.3%)	
RP595 <i>mraY1</i>	phospho-N-acetylmuramoyl-pentapeptide-transferase (B-Hin, 49.9%)	
RP825 <i>mraY2</i>	phospho-N-acetylmuramoyl-pentapeptide-transferase (B-Sac, 22.0%)	
RP807 <i>mraA</i>	penicillin binding protein 1A (B-Eco, 35.6%)	
RP579 <i>mraB</i>	UDP-N-acetylglucosamine 1-carboxyvinyltransferase (B-Eca, 51.5%)	
RP248 <i>mraB</i>	UDP-N-acetylglucosamine 1-carboxyvinyltransferase (B-Bsu, 35.7%)	
RP247 <i>mraC</i>	UDP-N-acetylmuramoylalanine ligase (B-Hin, 41.5%)	
RP410 <i>mraD</i>	UDP-N-acetylmuramoylalanine-D-glutamate ligase (B-Hin, 32.9%)	
RP597 <i>mraE</i>	UDP-MurNac-tripeptide synthetase (B-Bsu, 35.2%)	
RP596 <i>mraF</i>	UDP-MurNac-pentapeptide synthetase (B-Eco, 30.6%)	
RP412 <i>mraG</i>	UDP-MurNac-pentapeptide transferase (B-Bsu, 28.8%)	
RP565 <i>pbpA1</i>	penicillin binding protein (B-Hin, 34.3%)	
RP567 <i>pbpA2</i>	penicillin binding protein (B-Bsu, 30.7%)	
RP195 <i>pbpC</i>	penicillin-binding protein (B-Eco, 26.7%)	
RP259 <i>pbpE</i>	penicillin binding protein (B-Bsu, 25.2%)	
RP400 <i>sit</i>	lytic murein transglycosidase (B-Hin, 21.4%)	
<i>Surface polysaccharides, lipopolysaccharides and antigens</i>		
RP333 <i>capD</i>	capsular polysaccharide biosynthesis protein CapD (B-Sau, 34.6%)	
RP344 <i>capM1</i>	capsular polysaccharide biosynthesis protein CapM (B-Sau, 24.9%)	
RP414 <i>capM2</i>	capsular polysaccharide biosynthesis protein CapM prt (B-Sau, 23.7%)	
RP339 <i>ggaB</i>	galactosamine-containing teichoic acid biosynthesis (B-Bsu, 23.3%)	
RP178 <i>htrB</i>	lauroyl acetyltransferase (B-Hin, 21.5%)	
RP007 <i>lpxA</i>	UDP-N-acetylglucosamine acyltransferase (B-Rri, 90.4%)	
RP321 <i>lpxB</i>	lipid A disaccharide synthetase (B-Hin, 27.3%)	
RP254 <i>lpxC</i>	UDP-3-O-acetyl-N-acetylglucosamine deacetylase (B-Eco, 44.4%)	
RP009 <i>lpxD</i>	UDP-3-O-(R-3-hydroxymyristoyl)-glucosamine N-acetyltransferase (B-Rri, 92.4%)	
RP062 <i>kdsA</i>	3-deoxy-d-manno-octulosonic acid 8-phosphate synthetase (B-Pha, 45.1%)	
RP379 <i>kdsB</i>	CTP-CMP-3-deoxy-manno-octulosonate-cytidyl transferase (B-Hin, 34.8%)	
RP089 <i>kdtA</i>	3-deoxy-D-manno-octulosonic acid transferase (B-Eco, 28.9%)	
RP505 <i>kpsF</i>	polysialic acid capsule expression protein (B-Eco, 36.9%)	
RP833 <i>omp</i>	cell surface antigen, 17 kD (B-Rty, 46.9%)	
RP160 <i>omp1</i>	OMP1 precursor (B-Bab, 29.5%)	
RP771 <i>pal</i>	peptidoglycan-associated lipoprotein (B-Eco, 37.9%)	
RP476 <i>rfal</i>	lipopolysaccharide 1,2-glucosyltransferase (B-Sty, 26.1%)	
RP004 <i>rfbA</i>	O-antigen export system permease (B-Kpn, 22.3%)	
RP003 <i>rfbE</i>	O-antigen ABC export system, ATP-binding protein (B-Yen, 34.0%)	
RP334 <i>rfiE</i>	UDP-N-acetylglucosamine 2-epimerase (B-Bsu, 26.8%)	
RP018 <i>sca1</i>	cell surface antigen (B-Rri, 24.9%)	
RP081 <i>sca2</i>	cell surface antigen (B-Rri, 27.4%)	
RP451 <i>sca3</i>	cell surface antigen (B-Rri, 27.6%)	
RP498 <i>sca4</i>	cell surface antigen (B-Rja, 57.4%)	
RP704 <i>sca5</i>	cell surface antigen (B-Rti, 72.5%)	
RP779 <i>udg</i>	UDP-glucose 6-dehydrogenase (B-Pae, 31.8%)	
CELLULAR PROCESSES 44		
<i>Cell division</i>		
RP251 <i>ftsA</i>	cell division protein FtsA (B-Hin, 29.5%)	
RP043 <i>ftsH</i>	cell division protein FtsH (B-Eco, 54.0%)	
RP163 <i>ftsJ</i>	cell division protein FtsJ (B-Eco, 44.4%)	
RP823 <i>ftsK</i>	cell division protein FtsK (B-Cou, 41.5%)	
RP250 <i>ftsL</i>	cell division protein FtsL (B-Hin, 17.9%)	
RP411 <i>ftsW</i>	cell division protein FtsW (B-Eco, 33.2%)	
RP775 <i>ftsY</i>	cell division protein FtsY (B-Hin, 43.2%)	
RP666 <i>ftsZ</i>	cell division protein FtsZ (B-Wsp, 65.3%)	
RP056 <i>glfA</i>	glucose inhibited division protein A (B-Eco, 48.8%)	
RP057 <i>glfB</i>	glucose inhibited division protein (B-Ppu, 26.8%)	
RP815 <i>maf</i>	MAF protein (B-Bsu, 38.1%)	
RP042 <i>mesJ</i>	cell cycle protein MesJ (B-Eco, 22.1%)	
RP758 <i>mreB</i>	rod shape-determining protein (B-Eco, 60.5%)	
RP767 <i>mreC</i>	rod shape-determining protein (B-Eco, 23.1%)	
RP280 <i>rodA</i>	rod shape-determining protein (B-Eco, 38.1%)	
<i>Cell killing</i>		
RP555 <i>thyA</i>	hemolysin (B-Thy, 34.3%)	
RP740 <i>thyC</i>	hemolysin (B-Thy, 28.8%)	
<i>Chaperones and stress-induced proteins</i>		
RP670 <i>cspA</i>	cold shock protein (B-Sol, 57.6%)	
RP816 <i>dksA</i>	DnaK suppressor protein (B-Hin, 38.8%)	
RP184 <i>dnaJ</i>	heat shock protein (B-Eoy, 49.7%)	
RP185 <i>dnaK</i>	heat shock protein 70 (B-Rme, 72.7%)	
RP626 <i>groEL</i>	heat shock protein GroEL (B-Rme, 69.4%)	
RP627 <i>groES</i>	heat shock protein GroES (B-Rty, 98.9%)	
RP629 <i>grpE</i>	heat shock protein GrpE (B-Cor, 39.5%)	
RP200 <i>hscA</i>	heat shock protein A (B-Hin, 39.6%)	
RP320 <i>hslU</i>	heat shock protein HslU (B-Bsu, 54.8%)	
RP319 <i>hslV</i>	heat shock prt HslV (B-Hin, 54.1%)	
RP273 <i>hsp22</i>	heat shock protein (E-Hpy, cp, 29.0%)	
RP840 <i>hspG</i>	heat shock protein C62.5 (B-Eco, 43.1%)	
<i>Detoxification</i>		
RP535 <i>sodB</i>	superoxide dismutase (B-Lpn, 53.4%)	
RP759 <i>thdF</i>	thiophene and furan oxidizer (B-Hin, 34.7%)	
<i>Protein and peptide secretion</i>		
RP315 <i>aprD</i>	protease secretion ATP-binding protein (B-Pae, 40.0%)	
RP314 <i>aprE</i>	protease secretion ATP-binding protein (B-Pae, 32.4%)	
RP173 <i>flh</i>	signal recognition particle receptor protein (B-Eco, 49.6%)	
RP275 <i>lepA</i>	GTP-binding membrane protein (B-Bsu, 57.0%)	
RP116 <i>lepB</i>	signal peptidase (B-Sy, 37.3%)	
RP575 <i>secA</i>	preprotein translocase SecA subunit (B-Rca, 51.8%)	
RP070 <i>secB</i>	preprotein translocase SecB subunit (B-Hin, 30.7%)	
RP686 <i>secC</i>	preprotein-export membrane protein (B-Eco, 40.4%)	
RP134 <i>secE</i>	preprotein translocase SecE subunit (B-Bsu, 37.3%)	
RP114 <i>secF</i>	preprotein-export membrane protein (B-Hin, 37.7%)	
RP079 <i>secH</i>	preprotein-export membrane protein (B-Hpy, 32.0%)	
RP639 <i>secY</i>	preprotein translocase SecY subunit (B-Eco, 50.0%)	
RP842 <i>tig</i>	trigger factor (B-Eco, 32.0%)	
ENERGY METABOLISM 67		
<i>ATP-proton motive force interconversion</i>		
RP803 <i>atpA</i>	ATP synthase F1 alpha subunit (B-Rru, 66.2%)	
RP023 <i>atpB</i>	ATP synthase F0 subunit a (B-Rru, 51.5%)	
RP800 <i>atpC</i>	ATP synthase F1 epsilon subunit (B-Rru, 24.5%)	
RP801 <i>atpD</i>	ATP synthase F1 beta subunit (B-Rca, 77.0%)	
RP022 <i>atpE</i>	ATP synthase F0 subunit c (E-Ram mt, 43.2%)	
RP020 <i>atpF</i>	ATP synthase F0 subunit b (B-Rru, 21.1%)	
RP802 <i>atpG</i>	ATP synthase F1 gamma subunit (B-Rbl, 38.0%)	
RP804 <i>atpH</i>	ATP synthase F1 delta chain (E-Csl cp, 26.4%)	
RP021 <i>atpX</i>	ATP synthase F0 subunit b' (E-Ram mt, 28.6%)	
<i>Electron transport</i>		
RP588 <i>ccmE</i>	cytochrome c biogenesis protein (B-Hin, 33.2%)	
RP703 <i>ccmF</i>	cytochrome c biogenesis protein (E-Ram mt, 68.9%)	
RP405 <i>coxA</i>	cytochrome c oxidase subunit I (E-Mpo mt, 33.6%)	
RP406 <i>coxB</i>	cytochrome c oxidase subunit II (E-Mpo mt, 48.0%)	
RP191 <i>coxC</i>	cytochrome c oxidase subunit III (E-Pwi mt, 48.9%)	
RP257 <i>coxIV</i>	cytochrome c oxidase assembly (E-Sce, 35.2%)	
RP304 <i>cox11</i>	cytochrome c oxidase assembly (E-Ram mt, 42.9%)	
RP346 <i>ctaB</i>	cytochrome c oxidase assembly factor (B-Pde, 40.6%)	
RP253 <i>cyoM</i>	cytochrome c (B-Bja, 35.6%)	
RP216 <i>cyoD</i>	cytochrome oxidase d subunit I (B-Avi, 34.0%)	
RP217 <i>cyoB</i>	cytochrome oxidase d subunit II (B-Eco, 30.0%)	
RP272 <i>fbxH</i>	ubiquinol cytochrome c oxidoreductase, cytochrome c1 subunit (B-Bja, 47.8%)	
RP829 <i>fdxA</i>	ferredoxin (Rca, 57.5%)	
RP357 <i>nuoA</i>	NADH dehydrogenase I chain A (E-Pwi mt, 64.6%)	
RP356 <i>nuoB</i>	NADH dehydrogenase I chain B (E-Ram mt, 73.2%)	
RP355 <i>nuoC</i>	NADH dehydrogenase I chain C (B-Pde, 42.1%)	
RP354 <i>nuoD</i>	NADH dehydrogenase I chain D (E-Ram mt, 71.4%)	
RP353 <i>nuoE</i>	NADH dehydrogenase I chain E (E-Rno, mt, 55.1%)	
RP115 <i>nuoF</i>	NADH dehydrogenase I chain F (B-Pde, 69.1%)	
RP797 <i>nuoG</i>	NADH dehydrogenase I chain G (E-Bta mt, 49.3%)	
RP796 <i>nuoH</i>	NADH dehydrogenase I chain H (E-Ram mt, 63.5%)	
RP795 <i>nuoI</i>	NADH dehydrogenase I chain I (E-Ram mt, 71.4%)	
RP790 <i>nuoJ</i>	NADH dehydrogenase I chain J (E-Ram mt, 42.3%)	
RP791 <i>nuoK</i>	NADH dehydrogenase I chain K (B-Pde, 61.4%)	
RP792 <i>nuoL1</i>	NADH dehydrogenase I chain L (E-Ram mt, 45.5%)	
RP283 <i>nuoL2</i>	NADH dehydrogenase I chain L (E-Aja cp, 27.0%)	
RP282 <i>nuoL3</i>	NADH dehydrogenase I chain L (E-Ame mt, 17.0%)	
RP793 <i>nuoM</i>	NADH dehydrogenase I chain M (E-Ram mt, 46.6%)	
RP537 <i>nuoN1</i>	NADH dehydrogenase I chain N (E-Ram mt, 34.6%)	
RP284 <i>nuoN2</i>	NADH dehydrogenase I chain N (E-Ram mt, 23.2%)	
RP270 <i>petA</i>	Rieske-I iron sulphur protein (B-Bja, 58.3%)	
RP271 <i>petB</i>	cytochrome b (B-Rru, 65.4%)	
RP863 <i>pnIAA</i>	NAD(P) transhydrogenase α subunit (B-Eco, 37.7%)	
RP862 <i>pnIAB</i>	NAD(P) transhydrogenase α subunit (B-Hin, 44.7%)	
RP074 <i>pnIB</i>	NAD(P) transhydrogenase β subunit (B-Hin, 51.5%)	
<i>Fermentation</i>		
RP110 <i>ackA</i>	acetate kinase (B-Cts, 38.4%)	
<i>Glycolysis</i>		
RP492 <i>ppdK</i>	pyruvate, orthophosphate dikinase (E-Rfr, 48.8%)	
<i>Phosphate</i>		
RP589 <i>ppa</i>	inorganic pyrophosphatase (B-Eco, 59.3%)	
<i>Pyruvate dehydrogenase</i>		
RP261 <i>pdhA</i>	pyruvate dehydrogenase E1 component, α subunit (E-Ath, 44.0%)	
RP262 <i>pdhB</i>	pyruvate dehydrogenase E1 component, β subunit (E-Sce, 59.7%)	
RP530 <i>pdhC</i>	dihydrolipoyamide acetyltransferase E2 component (E-Rno, 45.1%)	
RP480 <i>pdhD</i>	dihydrolipoyamide dehydrogenase E3 component (E-Psa, 54.7%)	
RP805 <i>pdhE</i>	dihydrolipoyamide dehydrogenase E3 component (Zym, 51.1%)	
<i>TCa cycle</i>		
RP799 <i>aconA</i>	aconitate hydratase (B-Lpn, 59.1%)	
RP665 <i>fumC</i>	fumarate hydratase (B-Ror, 63.5%)	
RP844 <i>glfA</i>	citrate synthase (B-Rty, 97.8%)	
RP265 <i>icd</i>	isocitrate dehydrogenase (B-Tih, 38.6%)	
RP376 <i>mdh</i>	malate dehydrogenase (B-Can, 51.5%)	
RP128 <i>sdhA</i>	succinate dehydrogenase, flavoprotein subunit (B-Bja, 70.0%)	
RP044 <i>sdhB</i>	succinate dehydrogenase, iron-sulphur protein (E-Ram mt, 69.0%)	
RP126 <i>sdhC</i>	succinate dehydrogenase, cytochrome b ₅₅₆ subunit (E-Ram mt, 39.5%)	
RP127 <i>sdhD</i>	succinate dehydrogenase, subunit IV (E-Ram mt, 25.6%)	
RP180 <i>sucA</i>	2-oxoglutarate dehydrogenase (B-Hin, 44.3%)	
RP179 <i>sucB</i>	dihydrolipoyamide succinyltransferase (B-Eco, 48.7%)	
RP433 <i>sucC</i>	succinyl-CoA synthetase, β subunit (B-Eco, 52.1%)	
RP432 <i>sucD</i>	succinyl-CoA synthetase, α subunit (E-Dal, 70.7%)	
<i>Sugars</i>		
RP509 <i>exoC</i>	phosphomannomutase (B-Abr, 42.7%)	
RP299 <i>lacA</i>	galactosidase acetyltransferase (B-Mpn, 44.4%)	
FATTY ACID AND PHOSPHOLIPID METABOLISM 25		
RP620 <i>aas</i>	2-acyl-glycerol-phosphate-ethanolamine (B-Eco, 39.9%)	
RP038 <i>acoP1</i>	acyl-CoA desaturase (E-Yeast, 27.6%)	
RP763 <i>acoP2</i>	acyl carrier protein (B-Lmu, 52.6%)	
RP577 <i>acoP3</i>	holo-[acyl carrier protein] synthase (B-Eco, 38.5%)	
RP533 <i>blrA</i>	biotin Ac-CoA carboxylase synthase (B-Pde, 33.6%)	
RP424 <i>cysA</i>	phosphatidate cytidyltransferase (B-Eco, 31.5%)	
RP735 <i>fabD</i>	malonyl-CoA:acyl carrier protein transacylase (B-Bsu, 40.3%)	
RP764 <i>fabF</i>	3-oxoacyl-[acyl-carrier-protein] synthase II (B-Eco, 63.5%)	
RP762 <i>fabG</i>	3-oxoacyl-[acyl-carrier-protein] reductase (B-Rme, 54.8%)	

RP155 *pyrH* uridylylate kinase (B-Syn, 53.3%)

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RP229 *barA* histidine kinase sensor protein (B-Eco, 23.2%)
 RP471 *czcR* transcriptional activator protein (B-Aeu, 35.1%)
 RP426 *envZ* histidine kinase osmolarity sensor protein (B-Sil, 23.65%)
 RP294 *gppA* pppGpp phosphohydrolase (B-Hpy, 23.3%)
 RP011 *nirR* transcriptional activator nitrogen assimilation protein (B-Abr, 59.0%)
 RP614 *ntrY* histidine kinase nitrogen sensor protein (B-Aca, 30.6%)
 RP562 *ntrX* transcriptional activator nitrogen assimilation protein (B-Aca, 45.2%)
 RP427 *ompR* transcriptional activator protein OmpR (B-Rca, 42.9%)
 RP465 *phoR* histidine kinase phosphatase synthesis sensor protein (B-Bsu, 24.4%)
 RP312 *spoT** (p)ppGpp 3'-pyrophosphohydrolase (B-Eco, 29.9%)
 RP624 *spoT** (p)ppGpp 3'-pyrophosphohydrolase (B-Mpn, 27.8%)
 RP625 *spoT** (p)ppGpp 3'-pyrophosphohydrolase (B-Eco, 48.7%)
 RP705 *spoT** (p)ppGpp 3'-pyrophosphohydrolase (B-Eco, 31.7%)
 RP517 *yhbH* sigma 54 modulation protein (B-Bja, 26.2%)

REPLICATION 46

Degradation of DNA

RP734 *addA* ATP-dependent nuclease (B-Bsu, 23.7%)
 RP260 *xthA1* exodeoxyribonuclease III (B-Eco, 30.1%)
 RP676 *xthA2* exodeoxyribonuclease III (B-Eco, 33.2%)
 RP675 *xseA* exodeoxyribonuclease large subunit (B-Eco, 31.7%)
 RP350 *xseB* exodeoxyribonuclease small subunit (B-Eco, 32.5%)

DNA replication, restriction, modification, recombination and repair

RP601 *dnaA* chromosomal replication initiation protein DnaA (B-Eco, 44.1%)
 RP542 *dnaB* DNA helicase (E-Csi cp, 40.9%)
 RP778 *dnaB* DNA polymerase III alpha subunit (B-Sly, 37.2%)
 RP859 *dnaG* DNA primase (B-Sly, 29.0%)
 RP419 *dnaN* DNA polymerase III beta subunit (B-Ppu, 29.9%)
 RP732 *dnaX* DNA polymerase III epsilon subunit (B-Sly, 46.7%)
 RP605 *dnaX* DNA polymerase III gamma chain (B-Eco, 31.4%)
 RP206 *gyrA* DNA gyrase A subunit (B-Rsp, 49.4%)
 RP227 *gyrB* DNA gyrase B subunit (B-Sci, 42.0%)
 RP580 *gyrE2* DNA gyrase B subunit (B-Ppu, 51.5%)
 RP172 *holB* DNA polymerase II, delta prime subunit (B-Pae, 22.3%)
 RP171 *hupA* DNA binding protein HU (B-Vpr, 47.8%)
 RP720 *lig* DNA ligase (B-Zmo, 45.7%)
 RP777 *metK** S-adenosylmethionine synthetase (B-Eco, 66.3%)
 RP598 *mtf* transcription-repair coupling factor (B-Hin, 33.9%)
 RP351 *mgs* DNA-3-methyladenine glycosylase (E-Hsa, 29.7%)
 RP980 *mutL* DNA mismatch repair protein MutL (B-Spn, 35.4%)
 RP298 *mutS* DNA mismatch repair protein MutS (B-Bsu, 39.0%)
 RP746 *nth* endonuclease III (B-Eco, 50.7%)
 RP607 *parC* DNA topoisomerase IV subunit A (B-Hin, 39.0%)
 RP711 *pin** invertase/recombinase (B-Eco, 38.0%)
 RP776 *polA* DNA polymerase I (B-Bca, 37.2%)
 RP540 *prfA* primosomal protein replication factor (B-Rru, 39.7%)
 RP546 *radA* DNA repair (B-Bsu, 46.5%)
 RP761 *recA* recombination protein RecA (B-Pde, 71.2%)
 RP029 *recF* DNA repair protein, ATP binding protein (B-Cor, 30.4%)
 RP593 *recJ* ATP-dependent DNA helicase (B-Eco, 34.1%)
 RP528 *recK* single-stranded DNA-specific exonuclease (B-Eco, 32.8%)
 RP182 *recN* recombination protein RecN (B-Hin, 31.6%)
 RP438 *recR* recombination protein RecR (B-Bsu, 36.9%)
 RP385 *ruvA* Holliday junction DNA helicase (B-Pae, 35.0%)
 RP396 *ruvB* Holliday junction DNA helicase (Pae, 51.5%)
 RP119 *ruvC* Holliday junction endonuclease (B-Eco, 36.1%)
 RP386 *ssb* single-stranded binding protein (B-Bab, 52.6%)
 RP326 *topA* DNA topoisomerase I (B-Bsu, 44.9%)
 RP835 *uvrA* repair excision nuclease subunit A (B-Eco, 57.7%)
 RP203 *uvrB* repair excision nuclease subunit B (B-Hin, 56.0%)
 RP572 *uvrC* repair excision nuclease subunit C (B-Pfi, 36.9%)
 RP447 *uvrD* DNA helicase (B-Sau, 43.5%)
 RP817 *xerC* integrase/recombinase (B-Bsu, 32.2%)
 RP361 *xerD* integrase/recombinase (B-Eco, 37.6%)

TRANSCRIPTION 20

Degradation of RNA

RP504 *pnp* polyribonucleotide nucleotidyltransferase (B-Eco, 48.9%)
 RP117 *rnc* ribonuclease III (B-Hpy, 40.2%)
 RP462 *rnd* ribonuclease D (B-Eco, 28.5%)
 RP256 *rne* ribonuclease E (B-Eco, 35.9%)
 RP726 *rnhA* ribonuclease H1 (B-Msm, 43.4%)
 RP232 *rnhB* ribonuclease H1 (B-Eco, 44.7%)
 RP611 *rnpA* ribonuclease P (B-Mca, 28.4%)
 RP628 *rph* ribonuclease PH (B-Hin, 55.05%)

RNA synthesis and modification

RP861 *greA* transcription elongation factor GreA (B-Hin, 61.4%)
 RP553 *nusA* transcription termination factor NusA (B-Eco, 36.9%)
 RP162 *nusB* transcription termination factor NusB (B-Eco, 32.9%)
 RP135 *nusG* transcription antitermination protein NusG (B-Eco, 42.2%)
 RP015 *ponB* poly (A) polymerase I (B-Bsu, 26.3%)
 RP669 *rhlE* ATP-dependent RNA helicase (B-Eco, 38.3%)
 RP526 *rho* transcription termination factor Rho (B-Rsp, 72.0%)
 RP635 *rhoA* RNA polymerase alpha subunit (B-Bpe, 47.2%)
 RP140 *rhoB* RNA polymerase beta subunit (B-Rty, 87.4%)
 RP141 *rhoC* RNA polymerase beta subunit (B-Eco, 58.8%)
 RP303 *rhoH* RNA polymerase sigma-32 factor (B-Atu, 52.0%)
 RP858 *rhoD* RNA polymerase sigma-70 factor (B-Rca, 50.5%)

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Aminoacyl-tRNA synthetases

RP856 *alaS* alanyl-tRNA synthetase (B-Bba, 52.7%)
 RP065 *argS* arginyl-tRNA synthetase (B-Hpy, 33.0%)
 RP145 *aspS* aspartyl-tRNA synthetase (B-Syn, 43.3%)
 RP085 *cysS* cysteinyl-tRNA synthetase (B-Hin, 46.0%)
 RP325 *glxI1* glutamyl-tRNA synthetase (B-Abr, 45.6%)
 RP623 *glxX2* glutamyl-tRNA synthetase (B-Hpy, 40.3%)
 RP850 *glyC* glycyl-tRNA synthetase (B-Mca, 60.6%)
 RP489 *glyS* glycyl-tRNA synthetase (B-Bsu, 32.9%)
 RP308 *hisS* histidyl-tRNA synthetase (B-Eco, 38.3%)
 RP620 *ileS* isoleucyl-tRNA synthetase (B-Mtu, 48.6%)
 RP421 *leuS* leucyl-tRNA synthetase (B-Eco, 45.3%)
 RP371 *lysS* lysyl-tRNA synthetase (B-Bbu, 26.3%)
 RP683 *metS* methionyl-tRNA synthetase (B-Bsu, 46.9%)
 RP417 *pheS* phenylalanyl-tRNA synthetase alpha sub (B-Hin, 49.2%)
 RP418 *pheT* phenylalanyl-tRNA synthetase beta sub (B-Hin,

33.9%)
 RP384 *proS* proline-tRNA synthetase (B-Zmo, 51.8%)
 RP783 *serS* seryl-tRNA synthetase (B-Cbu, 47.2%)
 RP221 *thrS* threonyl-tRNA synthetase (B-Hin, 50.6%)
 RP468 *trpS* tryptophanyl-tRNA synthetase (B-Syn, 48.5%)
 RP556 *tyrS* tyrosyl-tRNA synthetase (B-Bca, 38.7%)
 RP687 *valS* valyl-tRNA synthetase (A-Mja, 38.3%)

tRNA and amino acyl-tRNA modification

RP238 *del* methionyl-tRNA deformylase (B-Eco, 49.4%)
 RP209 *fmt* methionyl-tRNA formyltransferase (B-Hin, 41.9%)
 RP152 *gatA* glutamyl-tRNA (Gln) amidotransferase subunit A (B-Mca, 48.6%)
 RP151 *gatB* glutamyl-tRNA (Gln) amidotransferase subunit B (B-Mca, 46.9%)
 RP153 *gatC* glutamyl-tRNA (Gln) amidotransferase subunit C (B-Bsu, 24.7%)
 RP672 *ksgA* dimethyladenosine transferase (B-Bsu, 35.7%)
 RP510 *miaA* tRNA delta-2-isopentenylpyrophosphate (IPP) transferase (B-Atu, 30.7%)
 RP605 *thi* peptidyl-tRNA hydrolase (B-Hin, 40.5%)
 RP213 *queA* S-adenosylmethionine:tRNA ribosyltransferase-iso merase (B-Hin, 43.3%)
 RP721 *tgt* tRNA-guanine transglycosylase (B-Zmo, 61.2%)
 RP111 *trmD* tRNA (guanine-N1)-methyltransferase (B-Eco, 44.7%)
 RP857 *truA* pseudouridylyl synthase I (B-Eco, 40.1%)
 RP501 *truB* tRNA pseudouridine 5S synthase (B-Hin, 37.6%)

Degradation of proteins, peptides and glycopptides

RP036 *clpB* ATP-dependent protease, ATP binding subunit (B-Hin, 54.3%)
 RP520 *clpP* ATP-dependent Clp protease (B-Yen, 67%)
 RP692 *clpX* ATP-dependent protease, ATPase subunit (B-Eco, 62.8%)
 RP228 *clp* tail-specific protease precursor (B-Bba, 42.6%)
 RP037 *gcp* sialoglycoprotein endopeptidase (B-Hin, 42.2%)
 RP123 *hflC* lambda cII stability-governing protein (B-Eco, 33.9%)
 RP122 *hflK* lambda cII stability-governing protein (B-Vpa, 30.3%)
 RP124 *htrA* serine protease (B-Bhe, 37.7%)
 RP186 *htrA* protease DO (E-Sce, 26.7%)
 RP450 *lon* ATP-dependent protease LA (B-Cor, 53.1%)
 RP408 *lspA* lipopeptide signal peptidase (B-Bsu, 27.9%)
 RP824 *map* methionyl aminopeptidase (B-Sly, 55.3%)
 RP219 *mpp* mitochondrial protease (B-Bsu, 35.4%)
 RP142 *pepA* aminopeptidase A (B-Pae, 36.6%)
 RP174 *ppcE* peptidase I1 (B-Rsn, 32.5%)
 RP281 *prfB* protease II (B-Eco, 34.2%)
 RP398 *sohB* protease IV (B-Mja, 23.9%)
 RP525 *sppA* protease IV (B-Hin, 27.6%)

Protein modification and translation factors

RP238 *elp* elongation factor P (B-Bsu, 39.5%)
 RP132 *ltaA* elongation factor G (B-Atu, 68.7%)
 RP184 *ltaB* initiation factor IF-1 (B-Hin, 67.1%)
 RP552 *infB* initiation factor IF-2 (B-Hin, 42.6%)
 RP531 *infC* initiation factor IF-3 (B-Ppi, 47.7%)
 RP529 *prfA* peptide chain release factor RF-1 (B-Bsu, 50.1%)
 RP274 *prfB* peptide chain release factor RF-2 (B-Eco, 50.4%)
 RP435 *rbfA* ribosome binding factor A (B-Bsu, 31.6%)
 RP693 *rimJ* ribosome protein alanine acetyltransferase (B-Eco, 20.2%)
 RP154 *rrf* ribosome recycling factor (B-Hin, 43.3%)
 RP397 *tlpA* thioldisulphide interchange protein (B-Bja, 27.4%)
 RP661 *tsf* elongation factor Tu (B-Tcu, 81.5%)
 RP087 *tsf* elongation factor Ts (B-Sci, 40.7%)

Ribosomal proteins; synthesis and modification

RP137 *rplA* ribosomal protein L1 (B-Cgr, 50.2%)
 RP656 *rplB* ribosomal protein L2 (E-Ram mt, 61.5%)
 RP659 *rplC* ribosomal protein L3 (E-Sce, 44.1%)
 RP658 *rplD* ribosomal protein L4 (B-Bst, 39.3%)
 RP647 *rplE* ribosomal protein L5 (B-Aco, 53.6%)
 RP644 *rplF* ribosomal protein L6 (B-Bst, 45.4%)
 RP041 *rplH* ribosomal protein L9 (E-Ppu cp, 33.6%)
 RP138 *rplJ* ribosomal protein L10 (B-Pfi, 36.7%)
 RP136 *rplK* ribosomal protein L11 (E-Ram mt, 45.5%)
 RP139 *rplL* ribosomal protein L7L12 (B-Bab, 66.9%)
 RP233 *rplM* ribosomal protein L13 (B-Sca, 52.8%)
 RP649 *rplN* ribosomal protein L14 (B-Eco, 69.6%)
 RP640 *rplO* ribosomal protein L15 (B-Bst, 46.5%)
 RP652 *rplP* ribosomal protein L16 (B-Aac, 53.3%)
 RP634 *rplQ* ribosomal protein L17 (B-Eco, 57.5%)
 RP643 *rplR* ribosomal protein L18 (B-Bst, 58.6%)
 RP112 *rplS* ribosomal protein L19 (B-Eco, 58.8%)
 RP609 *rplT* ribosomal protein L20 (B-Pey, 61.5%)
 RP751 *rplU* ribosomal protein L21 (E-Sci, 42.3%)
 RP654 *rplV* ribosomal protein L22 (B-Eco, 50.0%)
 RP657 *rplW* ribosomal protein L23 (B-Bst, 46.3%)
 RP648 *rplX* ribosomal protein L24 (B-Bst, 55.9%)
 RP606 *rplY* ribosomal protein L25 (B-Mtu, 26.9%)
 RP752 *rplA* ribosomal protein L27 (E-Ram mt, 62.9%)
 RP099 *rpmB* ribosomal protein L28 (B-Mge, 43.7%)
 RP651 *rpmC* ribosomal protein L29 (B-Bst, 59.4%)
 RP641 *rpmD* ribosomal protein L30 (B-Mtu, 33.3%)
 RP100 *rpmE* ribosomal protein L31 (B-Mtu, 31.6%)
 RP773 *rpmF* ribosomal protein L32 (B-Hin, 49.1%)
 RP879 *rpmG* ribosomal protein L33 (B-Hin, 51.8%)
 RP610 *rpmH* ribosomal protein L34 (B-Ppu, 65.9%)
 RP608 *rpmI* ribosomal protein L35 (E-Ppu cp, 38.5%)
 RP456 *rpmJ* ribosomal protein L36 (E-Gth cp, 65.8%)
 RP521 *rpsA* ribosomal protein S1 (B-Rme, 48.6%)
 RP086 *rpsB* ribosomal protein S2 (B-Mtu, 41.5%)
 RP653 *rpsC* ribosomal protein S3 (B-Bst, 54.2%)
 RP345 *rpsD* ribosomal protein S4 (B-Bsu, 43.2%)
 RP642 *rpsE* ribosomal protein S5 (B-Bst, 50.6%)
 RP309 *rpsF* ribosomal protein S6 (B-Hin, 30.2%)
 RP131 *rpsG* ribosomal protein S7 (E-Ram mt, 45.2%)
 RP645 *rpsH* ribosomal protein S8 (B-Bsu, 42.0%)
 RP234 *rpsI* ribosomal protein S9 (B-Bst, 48.8%)
 RP660 *rpsJ* ribosomal protein S10 (B-Hin, 60.8%)
 RP636 *rpsK* ribosomal protein S11 (B-Syn, 53.5%)
 RP130 *rpsL* ribosomal protein S12 (B-Tth, 60.5%)
 RP637 *rpsM* ribosomal protein S13 (B-Bst, 58.8%)
 RP646 *rpsN* ribosomal protein S14 (B-Syn, 47.0%)
 RP503 *rpsO* ribosomal protein S15 (B-Bst, 53.4%)
 RP878 *rpsP* ribosomal protein S16 (B-Hin, 45.1%)
 RP650 *rpsQ* ribosomal protein S17 (B-Tth, 61.8%)
 RP040 *rpsR* ribosomal protein S18 (B-Syn, 50.7%)
 RP655 *rpsS* ribosomal protein S19 (B-Eco, 58.2%)
 RP633 *rpsT* ribosomal protein S20 (B-Rme, 40.9%)
 RP615 *rpsU* ribosomal protein S21 (B-Bbu, 33.3%)

TRANSPORT AND BINDING PROTEINS 38

General

RP060 *abcT1* ABC transporter, ATP-binding protein (B-Hin, 55.7%)
 RP508 *abcT2* ABC transporter, ATP-binding protein (B-Rme,

48.8%)

RP214 *abcT3* ABC transporter, ATP-binding protein (B-Hin, 33.6%)
 RP387 *msbA1* ABC transporter, ATP-binding protein (B-Eco, 26.2%)
 RP696 *msbA2* ABC transporter, ATP-binding protein (B-Eco, 28.2%)

Amino acids

RP307 *alcT* cationic amino acid transporter (E-Mmu, 29.7%)
 RP129 *glnP* glutamine transport system permease (B-Bsu, 48.6%)
 RP700 *glnQ1* glutamine ABC transporter, ATP-binding protein (B-Eco, 39.1%)
 RP868 *glnQ2* glutamine ABC transporter, ATP-binding protein (B-Bst, 51.0%)
 RP176 *gltP* glutamate-aspartate transporter (B-Bca, 35.2%)
 RP483 *potE* putrescine-ornithine transporter (B-Hin, 26.9%)
 RP369 *potG* putrescine ABC transporter, ATP-binding protein (B-Mpn, 29.2%)
 RP077 *proP1* proline/betain transporter (B-Eco, 26.7%)
 RP313 *proP2* proline/betain transporter (B-Eco, 24.9%)
 RP375 *proP3* proline/betain transporter (B-Eco, 21.2%)
 RP685 *proP4* proline/betain transporter (B-Eco, 24.0%)
 RP755 *proP5* proline/betain transporter (B-Eco, 27.8%)
 RP852 *proP6* proline/betain transporter (B-Eco, 34.8%)
 RP881 *proP7* proline/betain transporter (B-Eco, 28.7%)
 RP150 *yqjX* amino acid ABC transporter (B-Bsu, 32.4%)

Nucleosides and nucleotides

RP097 *mkl* ribonucleotide ABC transporter, ATP-binding protein (B-Mle, 36.2%)
 RP053 *tlc1* ATP/ADP translocase (B-Ctr, 43.3%)
 RP377 *tlc2* ATP/ADP translocase (B-Ctr, 35.2%)
 RP477 *tlc3* ATP/ADP translocase (B-Ctr, 39.6%)
 RP500 *tlc4* ATP/ADP translocase (B-Ctr, 36.3%)
 RP739 *tlc5* ATP/ADP translocase (B-Ctr, 34.7%)

Carbohydrates, organic alcohols and acids

RP054 *glpT* glycerol-3-phosphate permease (B-Bsu, 37.1%)

Cations

RP834 *afuC* iron ABC transporter, ATP-binding protein (B-Eco, 33.9%)
 RP810 *kefB* glutathione-regulated potassium-efflux system protein (B-Eco, 33.9%)
 RP583 *mgfE* magnesium transporter (B-Syn, 27.0%)

Other

RP205 *atm1* mitochondrial ABC transporter, ATP-binding protein (E-Sce, 43.3%)
 RP794 *ccmA* haem ABC transporter A, ATP-binding protein (B-Hin, 35.5%)
 RP268 *ccmB* haem exporter protein B (E-Ram mt, 20.9%)
 RP830 *ccmC* haem exporter protein C (B-Bja, 43.7%)
 RP571 *panF* panthotenate permease (B-Hin, 20.5%)
 RP630 *perM* permease PerM homologue (B-Hin, 25.0%)
 RP374 *secT* transport protein SecT (B-Hsa, 29.6%)
 RP576 *prsA* protein export (B-Bsu, 28.9%)

OTHER CATEGORIES 52

Adaptations to atypical conditions

RP708 *himA* integration host factor α (B-Eco, 29.5%)
 RP236 *invA* invasion protein A (B-Bba, 42.8%)
 RP590 *mviN* virulence factor MviN protein (B-Sly, 32.4%)
 RP717 *taxX** conjugative DNA processing (B-Eco, 33.5%)
 RP286 *trgB* conjugal transfer (B-Rsn, 24.7%)
 RP103 *virB4* virulence protein VIRB4 (B-Atu, 30.9%)
 RP784 *virB4* virulence protein VIRB4 (B-Atu, 20.3%)
 RP287 *virB8* virulence protein VIRB8 (B-Atu, 20.4%)
 RP290 *virB9* virulence protein VIRB9 (B-Atu, 24.8%)
 RP291 *virB10* virulence protein VIRB10 (B-Atu, 20.3%)
 RP292 *virB11* virulence protein VIRB11 (B-Atu, 29.6%)
 RP293 *virD4* virulence protein VIRD4 (B-Atu, 31.3%)

Drug and analogue sensitivity

RP170 *acrD* acriflavin resistance protein D (B-Eco, 31.3%)
 RP475 *ampG1AMPG* protein (B-Eco, 31.4%)
 RP668 *ampG2AMPG* prt (B-Eco, 26.3%)
 RP781 *ampG3AMPG* prt (B-Eco, 27.6%)
 RP603 *bcr1* bicyclomycin resistance (B-Eco, 21.7%)
 RP698 *bcr2* bicyclomycin resistance (B-Eco, 18.8%)
 RP243 *emrA* multidrug resistance protein A (B-Eco, 26.9%)
 RP157 *emrB* multidrug resistance protein B (B-Hin, 29.3%)
 RP786 *terC* tellurite resistance protein (B-Eco, 35.3%)

Colicin-related functions

RP302 *tolB* colicin tolerance protein (B-Hin, 29.8%)
 RP309 *tolC* inner membrane protein (B-Eco, 39.7%)
 RP310 *tolR* inner membrane protein (B-Pae, 40.1%)

Uncatalogued

RP493 *addA* adducin alpha subunit (E-Hsa, 32.6%)
 RP199 *adx1* adrenodoxin precursor (E-Spo, 57.1%)
 RP714 *ank2* ankyrin (B-Eco, 32.7%)
 RP245 *bolA* BolA protein (B-Val, 34.2%)
 RP181 *ctaQ** thermostable carboxypeptidase (B-Pho, 29.1%)
 RP297 *cysG* sulphite synthesis pathway protein (B-Eco, 31.2%)
 RP323 *cyaY* CyaY protein (B-Ech, 31.1%)
 RP118 *era* GTP binding protein Era (B-Bsu, 33.6%)
 RP063 *hesB1* HesB protein (B-Ava, 37.0%)
 RP484 *hesB2* HesB protein (B-Pbo, 40.2%)
 RP212 *n2B* N25, ATase protein (E-Hir, 27.2%)
 RP485 *nifU* nitrogen fixation protein (B-Avi, 43.0%)
 RP832 *p34* p34 protein (B-Rri, 91.3%)
 RP602 *pat1* patatin B1 precursor protein (E-Stu, 22.9%)
 RP317 *pkcl* protein kinase C inhibitor (B-Abr, 38.6%)
 RP109 *ptb* phosphate butyryltransferase (B-Cab, 36.4%)
 RP594 *scoB** succinyl-CoA:3-ketoacid-CoA transferase subunit B (B-Mtu, 22.0%)
 RP031 *sco2* Sco yeast precursor protein (E-Sce, 32.6%)
 RP687 *sco2* Sco yeast precursor protein (E-Sce, 36.6%)
 RP946 *shB* ShB protein (B-Zmo, 40.6%)
 RP430 *smfB* small protein (B-Syn, 48.7%)
 RP058 *soj* SOJ protein (B-Bsu, 50.4%)
 RP486 *sp1* tRNA splicing protein (E-Cal, 58.9%)
 RP487 *sp1* tRNA splicing protein (E-Cal, 32.3%)
 RP059 *spoJ1* sporulation protein (B-Bsu, 40.4%)
 RP239 *suH8* suppressor protein (B-Eco, 22.8%)
 RP733 *surF1* SurF1 protein (E-Hsa, 23.9%)
 RP710 *tra3** transposase (B-Rme, 34.0%)

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Discovery of a young nearby supernova remnant

Bernd Aschenbach

Max-Planck-Institut für extraterrestrische Physik, D-85740 Garching, Germany

About 200 supernova remnants have been found in the galaxy¹, six of which are younger than about 1,000 years (ref. 2). Observations of these young remnants are important for understanding of the late phases of supernova evolution, and each new object should add substantially to our knowledge of the processes involved. Here I report the discovery of a supernova remnant (RX J0852.0 – 4622), identified by its X-ray emission, at the southeast corner of the known Vela supernova remnant. The high temperature ($> 3 \times 10^7$ K) indicates an age of less than ~1,500 yr. The observed diameter of the remnant is about 2° , which suggests a distance of less than 1 kpc, based on a comparison with the remnant of the supernova of AD 1006. RX J0852.0 – 4622 may therefore be the nearest supernova to have occurred during recent human history.

The Vela region was mapped in soft X-rays by Rosat in 1990/91. The image obtained for photon energies $E > 0.1$ keV is shown in Fig. 1, left panel. When I used energy bands different from the Rosat standard settings, I discovered an X-ray source which becomes visible only at $E > 1.3$ keV (Fig. 1, right panel) but which is outshone otherwise by the emission of the Vela supernova remnant (SNR). The source, located at the southeast corner of the Vela SNR,

displays a circular emission region with a diameter of 2° centred on right ascension (2000) 8 h 52 min 3 s, declination (2000) $-46^\circ 22'$, which was accordingly named RX J0852.0 – 4622. It is limb brightened in the north and south with a limb width of ~20% of the source radius; the morphology suggests the source to be a previously unknown SNR¹. No radio counterpart^{3,4} showing the same full size has been found, but the northern limb appears to coincide with the 408 MHz emission peak of Vela Z, which is a non-thermal radio source⁵. Recently Vela Z has been mapped and spatially resolved in the 843 MHz MOST survey⁶. Vela Z shows a relatively broad arc-like structure 40 arcmin long, which matches the brightest section of the northern limb of RX J0852.0 – 4622 in position and shape. The association of Vela Z and RX J0852.0 – 4622 supports the identification of the latter as an SNR.

The analysis of the X-ray spectra shows that the emission from the northern limb of RX J0852.0 – 4622 may be thermal with temperatures $T_{1,1}$ and $T_{1,2}$; however, it can also be represented by a power-law spectrum created by synchrotron radiation. In contrast the emission from the rest of the remnant (excluding the northern and the southern limb sections) is clearly of thermal origin with temperatures $T_{r,1}$ and $T_{r,2}$. Independent of whether the northern limb emission is thermal or non-thermal, RX J0852.0 – 4622 is a young SNR because the temperatures observed are high. As heating occurs by shock waves the temperatures $T_{r,2}$ and possibly $T_{1,2}$ correspond to shock wave velocities of $1,500 \text{ km s}^{-1}$ and $2,000 \text{ km s}^{-1}$, respectively. Such high internal velocities have so far been observed only in SNRs younger than ~1,500 years (ref. 2).

X-ray synchrotron emission from two limb sectors and thermal emission from the rest of the remnant of SN1006 has been

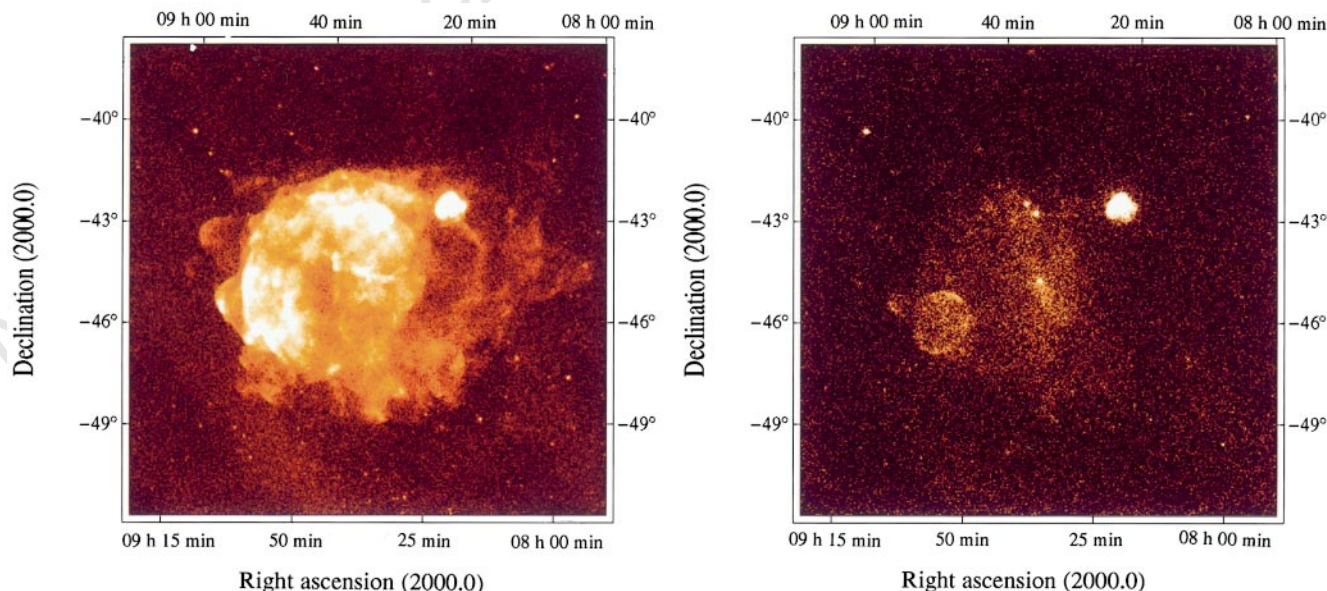


Figure 1 Rosat all-sky survey images of the Vela SNR and its surroundings. Angular resolution is 1 arcmin half-power radius; mean exposure is 993 s. The left-hand image was taken for photon energies $0.1 < E < 2.4$ keV; surface brightness increases from dark yellow to white by a factor of 500. The right-hand image is for photon energies > 1.3 keV. Most of the Vela SNR X-ray emission which dominates at low energies had disappeared. At the centre, the synchrotron nebula around the Vela pulsar remains visible as well as the SSW beam-like structure, and at the very northwest (upper right) the bright Puppis-A SNR can be seen. The new shell-type SNR RX J0852.0 – 4622 shows up in the lower left. East of RX J0852.0 – 4622 hard X-ray photons from the D/D' Vela SNR shrapnels are seen which, however, are associated with a much lower-temperature spectrum than RX J0852.0 – 4622 (ref. 14). For X-ray spectral analysis, RX J0852.0 – 4622 was divided into two

regions, one containing the bright northern limb section (l) and the other one (r) excluding the northern and southern limbs. Spectral fits were performed with either power-law models, optically thin thermal emission equilibrium models (Raymond-Smith models) or combinations of both. Solutions with a reduced $\chi^2 < 1$ for region r are obtained only with a two-temperature model with $kT_{r,1} = 0.14^{+0.08}_{-0.03}$ keV, $kT_{r,2} = 2.5^{+4.5}_{-0.7}$ keV. The spectrum of the northern limb can be fitted by either a simple power law with index $\alpha = -2.6^{+0.3}_{-0.4}$ or a two-temperature model with $kT_{l,1} = 0.21^{+0.14}_{-0.09}$ keV, $kT_{l,2} = 4.7^{+4.5}_{-0.7}$ keV. The presence of low-temperature components may partially be due to a residual, uncorrected contribution from the much softer Vela SNR. The total, absorption-corrected flux of the high-temperature components is $F_x(0.1\text{--}2.4 \text{ keV}) = 3 \times 10^{-10} \text{ erg cm}^{-2} \text{ s}^{-1}$.

reported^{7,8}; the spectra of RX J0852.0 – 4622 are similar to these if the power-law-like spectrum of its northern limb section is applied. The X-ray spectral parameters which Willingale *et al.*⁸ have obtained for SN1006 and our results for RX J0852.0 – 4622 are basically the same concerning the spectral index α as well as the temperatures $T_{r,1}$ and $T_{r,2}$ of the SNR interior. Furthermore, the X-ray morphology of the two objects is very similar for $E > 1$ keV, they have the same surface brightness for the thermal region within a factor of 1.3. The non-thermal northern limb of SN1006, however, is brighter by a factor of 20. I note that the ratio of energy flux density at 843 MHz (~ 10 mJy arcmin⁻²; D. Bock, personal communication) to that at 1 keV (1.2×10^{-8} Jy arcmin⁻²) of 8×10^5 is close to that of SN1006 which is 7×10^5 (ref. 9), supporting a synchrotron model but with significantly less non-thermal energy density. Either of the two brightness ratios can be used to estimate the distance d to RX J0852.0 – 4622, assuming brightness proportional to $(\Phi d)^{-3}$, which holds for similar ambient conditions, that is either thermal electron density or relativistic electron density times magnetic energy density. With an angular diameter Φ four times bigger, RX J0852.0 – 4622 is closer by a factor of 1.5–4, which is $d = 500$ pc to 1.4 kpc for $d_{\text{SN1006}} = 2$ kpc (ref. 10). If d_{SN1006} is as low as 800 pc (ref. 8), d is reduced to 200–600 pc. Large distances (> 1 kpc) can be excluded by the 5σ upper limit of the photoelectrical absorption column density N_{H} of 8×10^{21} cm⁻² observed in the X-ray spectra. This limit requires RX J0852.0 – 4622 to lie in front of the Vela molecular ridge, the distance of which is ~ 1 kpc (ref. 11). From the size and X-ray flux observed in the high-temperature components, an upper limit to the density of the ambient medium in which the progenitor of RX J0852.0 – 4622 exploded can be given as $0.04d_5^{-0.5}$ cm⁻³ (with d_5 measured in units of 500 pc). This low external density, as well as the low age (which limits the electron acceleration time⁹) may be responsible for the low level of X-ray synchrotron radiation.

I have searched the Rosat all-sky survey data of RX J0852.0 – 4622 for a point-like, compact, stellar remnant. The 5σ upper limit of the 0.1–2.4 keV flux $F_{5\sigma}$ corrected for absorption with $N_{\text{H}} = 2 \times 10^{20}$ cm⁻² is 3×10^{-12} erg cm⁻² s⁻¹ anywhere except in a 3 arcmin by 3 arcmin region centred on RA(2000) = 8 h 52 min 3 s, Dec(2000) = -46.31° , where an excess count rate F_{point} of 8×10^{-12} erg cm⁻² s⁻¹ was found. But this field includes the B8 IV/Vstar HD76060 with visual magnitude $m_v = 7.9$ and the Be star IRAS08502 – 4606 with $m_v = 13.8$. If the supernova left a pulsar, it is less luminous than the Crab pulsar by about four orders of magnitude for $d_5 = 1$, provided that the pulsar beam crosses the line of sight. A young neutron star is supposed to be hot, radiating X-rays from the black-body surface with a flux (in the 0.1–2.4 keV range) of $F_{\text{ns}} = (2 \times 10^{-10} \text{ erg cm}^{-2} \text{ s}^{-1}) R_{10}^2 T_{1,7}^4 d_5^{-2}$, with R_{10} the neutron-star radius in units of 10 km and $T_{1,7}$ the surface temperature in units of 1.7×10^6 K, which is expected from standard cooling models for a neutron star $\sim 1,000$ years old¹². The values of $F_{5\sigma}$ and F_{point} seem to rule out the presence of such a neutron star in RX J0852.0 – 4622 by a large factor; however, a neutron star of somewhat lower temperature and smaller radiating surface area could be there. This result does not therefore exclude the possibility that RX J0852.0 – 4622 is the remnant of a core-collapse supernova, but leaves unanswered the question about the supernova type. $\square$

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Correspondence and requests for materials should be addressed to the author (e-mail: bra@mpe-garching.mpg.de).

Emission from ⁴⁴Ti associated with a previously unknown Galactic supernova

A. F. Iyudin*, V. Schönfelder*, K. Bennett†, H. Bloemen‡, R. Diehl*, W. Hermsen‡, G. G. Lichti*, R. D. van der Meulen‡, J. Ryan§ & C. Winkler†

* Max Planck Institut für extraterrestrische Physik, Postfach 1603, D-85740 Garching, Germany

† Astrophysics Division, ESTEC, 2200 AG Noordwijk, The Netherlands

‡ SRON-Utrecht, Sorbonnelaan 2, 3584 CA Utrecht, The Netherlands

§ University of New Hampshire, Institute for Studies of Earth, Oceans and Space, Durham, New Hampshire 03824, USA

Nearly 400 years have passed since a supernova was last observed directly in the Milky Way (by Kepler, in 1604). Numerous Galactic supernovae are expected to have occurred since then¹, but only one (Cassiopeia A) may have been seen². The historical record of supernovae is therefore incomplete, as demonstrated by the spatial distribution of young supernova remnants³. The discovery^{4,5} of γ -ray emission from the decay of ⁴⁴Ti nuclei associated with Cassiopeia A, the youngest known remnant, has revealed a new way to search for the remnants of other relatively recent supernovae (less than $\sim 1,000$ years old). Here we report the discovery of ⁴⁴Ti line emission from a previously unknown young supernova remnant, in the direction of the Vela remnant. We estimate a distance of ~ 200 parsecs and an age of ~ 680 years for the remnant, making it the closest young remnant to the Earth. Why it was not recorded historically remains unknown.

⁴⁴Ti is expected to be produced in different types of supernovae^{6–9}, although with very different abundances (typically $\sim 7 \times 10^{-5} M_{\odot}$ by type Ia⁶; up to $10^{-4} M_{\odot}$, depending on the mass of the condensed star-like remnant, for the most frequent types II and Ib^{7,8}; and up to $3.9 \times 10^{-3} M_{\odot}$ for the rare helium white-dwarf detonation⁹; here $M_{\odot}$ is the solar mass). The Galaxy is almost transparent in the γ -ray band, unlike the situation for optical wavelengths, so that hitherto optically obscured supernovae may become detectable at γ -ray energies. For this purpose, the γ -ray line from the decay-chain of ⁴⁴Ti at 1.156 MeV is very suitable. Because of the ⁴⁴Ti lifetime of ~ 90 yr, this line is the best indicator of young (age $\leq 1,000$ yr) Galactic supernova remnants (SNRs)¹⁰. In addition, the decay time is comparable to the average time interval between Galactic supernovae, so ⁴⁴Ti γ -ray line emission should appear as localized sources rather than diffuse emission.

The first detection (that of Cas A) was obtained by COMPTEL⁴. This youngest Galactic SNR was seen in the early phase of the CGRO

mission at the $\sim 4.1\sigma$ level⁴ and was confirmed later at a higher significance level (5.2σ) in a 5-year accumulation of COMPTEL data⁵. This detection of Cas A has proved that ^{44}Ti was synthesized and ejected in core-collapsed supernovae, and has indicated the feasibility of using the 1.156-MeV ^{44}Ti line for uncovering young Galactic SNRs. A previous search for Galactic sources of ^{44}Ti γ -ray line emission based on a 3-year accumulation of data was not conclusive in finding new excesses, apart from the already known detection of Cas A¹¹. In comparison, the present survey is using much more data (better exposure) covering the time interval from April 1991 to October 1997. In addition to Cas A, one more significant source of ^{44}Ti line emission was found (GRO J0852–4642). Presumably we have detected ^{44}Ti line emission from a young Galactic SNR that was missed by the optical and radio surveys. A description of the main properties of COMPTEL, of its range of data and of the line-emission image and spectral analysis have been presented elsewhere^{4,5,12–15}.

A maximum-likelihood method was used for the image analysis in the energy range 1.066–1.246 MeV (that is, $\pm 2\sigma$ around the ^{44}Ti line energy) in order to search for point sources. Figure 1 shows such a map of the Vela region with an excess of the line emission. The likelihood ratio at the position of the excess is $-2\ln\lambda = 38.3$, which is equivalent to a 5.6σ detection (3 degrees of freedom). The line flux from the new SNR derived by the imaging analysis for the combination of all data is $(3.8 \pm 0.7) \times 10^{-5} \text{ cm}^{-2} \text{ s}^{-1}$. We estimate that this flux could be subject to a systematic error of $\pm 20\%$.

A spectral analysis of the GRO J0852–4642 γ -ray emission was performed for the 32 observations with the best exposure by selecting events from a circular region of 2° radius centred at the

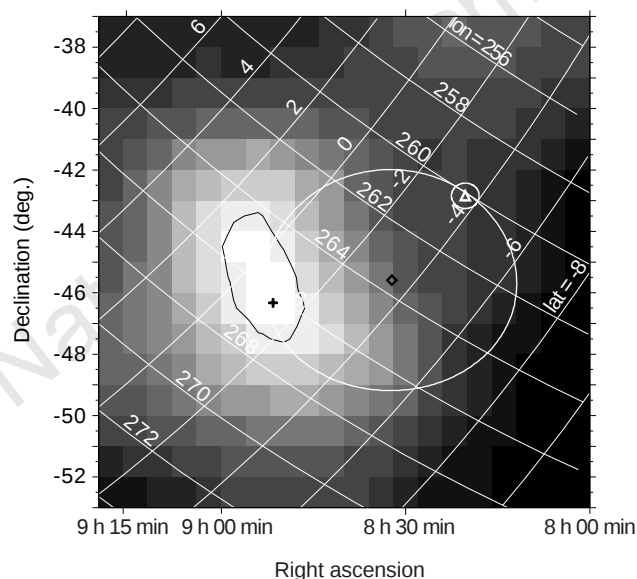


Figure 1 COMPTEL maximum-likelihood map of the Vela region in the energy range 1.066–1.246 MeV. This energy range is $\pm 2\sigma$ around the ^{44}Ti line energy. The cross marks the position of the possible counterpart RX J0852.0–4622, seen at X-ray energies²⁰. The likelihood ratio at the position of GRO J0852–4642 (Galactic coordinates $l = 266.5^\circ$, $b = -1.5^\circ$) is $-2\ln\lambda = 38.3$ (for the combination of all data), which is equivalent to a 5.6σ detection (3 degrees of freedom). A 1σ position error contour is shown in black. For the same combination of the data, in an all-sky search, the probability of finding one random excess with a $-2\ln\lambda$ value of ~ 38.3 is $< 9.4 \times 10^{-6}$ for 470 trials. The diamond marks the position of the Vela pulsar, and the triangle marks the centre of the Puppis A SNR. The approximate sizes of Vela SNR and Puppis A are shown by white circles. Right ascension and declination are in the J2000 system; the Galactic coordinates grid is overlaid.

position of the ^{44}Ti excess, and background spectra were constructed using three different methods. These yield similar results, giving a final significance for the ^{44}Ti line in the residual spectra ranging from 5.2σ to 7σ (see Fig. 2). Two lines are clearly seen, at 1.16 MeV and 1.8 MeV. The 1.8-MeV line from this region (due to ^{26}Al) was detected by COMPTEL earlier in the mission¹⁶. The ^{26}Al emission in the Vela SNR region is thought to be produced in an extended region^{13,15–17}. The second line in the spectrum at 1.16 MeV is the main discovery of the ^{44}Ti all-sky survey. From the analysis of the residual spectra with larger-radius ($\geq 3^\circ$) windows around the ^{44}Ti excess position, we found an increase in the significance of the 1.8 MeV-line for larger windows, and at the same time a decrease of the 1.16-MeV line significance. This is consistent with the 1.16-MeV line origin from a point-like source, whereas the 1.8-MeV line originates in an extended region, in agreement with previous conclusions^{13,15–17}.

The mean energy of this 1.16-MeV line derived from the summed spectrum (Fig. 2) is $(1,163 \pm 16) \text{ keV}$, consistent with the ^{44}Ti origin. The width of the fitted ^{44}Ti line ($\sigma \approx 71 \text{ keV}$) was found to be larger than the energy resolution of COMPTEL at 1.16 MeV ($\sigma_{\text{instr.}} \approx 45 \text{ keV}$). This may indicate that the ^{44}Ti line is Doppler-broadened. But the 1.8-MeV line also appears somewhat broader (for the point-source and for the extended-region spectra) than the instrumental resolution (80 keV, compared to $\sim 59 \text{ keV}$); this might point either to a deterioration of the telescope energy resolution with time, or alternatively to Doppler broadening. If the broadening of the ^{44}Ti line was due to the motion of the ejecta, then a velocity of $\sim 15,300 \pm 3,700 \text{ km s}^{-1}$ is derived for the ejecta, which in our opinion should be treated as an upper limit.

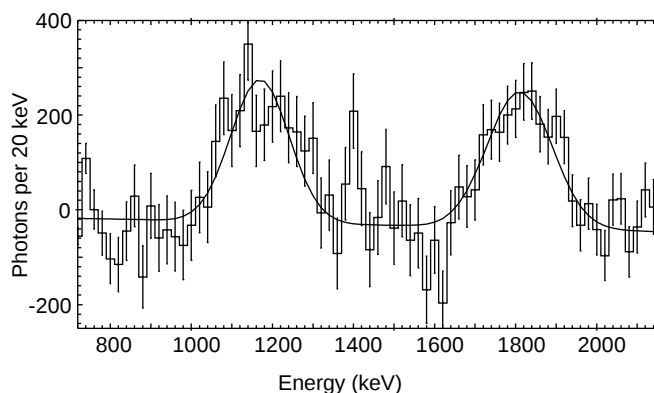


Figure 2 As example of the background-subtracted spectra from the 2° -radius circle centred on Galactic coordinates $l = 266.5^\circ$, $b = -1.5^\circ$ for a combination of 32 observations. Two lines are clearly seen, at 1.16 MeV and 1.8 MeV, with significances of $\sim 7\sigma$ and $\sim 8\sigma$, respectively. The mean energy of the 1.16-MeV line derived from this spectrum is $1,163 \pm 16 \text{ keV}$, with an r.m.s. width of $\sigma = (71 \pm 12) \text{ keV}$, consistent with ^{44}Ti origin. A linear residual background and two Gaussian-shaped lines were fitted, with 8 free parameters in total (χ^2 is 137.3 for 92 degrees of freedom). The background spectrum used is based on a physical model of the instrument, which takes into account two prominent background lines at 1.46 MeV (arising from the radioactive ^{40}K decay in the PMT glass housing of the upper detector¹⁴) and at 2.223 MeV (arising from thermal-neutron capture on hydrogen in these detectors). A third line was included to account for a rather broad and much weaker background feature between 1.3 and 1.7 MeV, produced by the ^{22}Na build-up in the instrument¹⁵.

The line flux from the SNR can be translated into an ejected mass of ^{44}Ti , if we know the age and distance of the SNR. Recent measurements of the ^{44}Ti lifetime (see refs 18, 19, and references therein) were used to derive a weighted mean of 90.4 ± 1.3 years. We note that the effective ^{44}Ti lifetime in SNRs could be larger, depending on the degree of ionization of the ^{44}Ti and its Lorentz factor. The derived value of the ejected ^{44}Ti mass is mainly sensitive to the actual value of the lifetime and is less critically dependent on the distance to, and the age of, the SNR.

The parameters (age and distance) are not available from the γ -ray measurements alone. Fortunately however, a possible counterpart of the newly discovered SNR was recently (independently) detected in Rosat data²⁰: an extended feature of $\sim 2^\circ$ diameter centred at Galactic longitude $l = 266.3^\circ$, latitude $b = 1.2^\circ$ only 0.4° away from the ^{44}Ti excess, well within the measurement uncertainties.

By combining the γ -ray line flux and the X-ray diameter²⁰ with an assumed typical ^{44}Ti yield of $\sim 5 \times 10^{-5} M_\odot$ for supernovae of different types^{6–9}, and taking as representative an expansion velocity of $\sim 5,000 \text{ km s}^{-1}$ (ref. 21) for the supernova ejecta, we derive a distance of ~ 200 pc, and an age of the SNR of ~ 680 yr. For larger ^{44}Ti yields and larger expansion velocities, the distance estimate becomes larger and the age estimate becomes less. We note that the SNR expansion velocity, when evaluated from the SNR X-ray spectrum²⁰, has the same value of $\sim 5,000 \text{ km s}^{-1}$.

We can only speculate about the reasons why this supernova was not observed ~ 700 years ago: we can consider the possible existence both of optically subluminal supernovae²² and of absorbing material in front of the supernova. In addition, the celestial position and the time of the event might have been unfavourable for an observation. Information about the existence and type of the compact stellar-like remnant of the supernova, and the elemental abundances of the SNR, will have to await future optical, radio, X-ray and γ -ray measurements. $\square$

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Correspondence and requests for materials should be addressed to A.F.I. (e-mail: ani@mpe-garching.mpg.de).

Paramagnetic Meissner effect in small superconductors

A. K. Geim*, S. V. Dubonos†, J. G. S. Lok*, M. Henini‡ & J. C. Maan*

* Research Institute for Materials, University of Nijmegen, 6525 ED Nijmegen, The Netherlands

† Institute for Microelectronics Technology, Russian Academy of Sciences, 142432 Chernogolovka, Russia

‡ Department of Physics, University of Nottingham, Nottingham NG7 2RD, UK

A superconductor placed in a magnetic field and cooled down through the transition temperature expels magnetic flux. This phenomenon, known as the Meissner effect, is arguably the most essential property of superconductors and implies zero resistivity. Surprisingly, several recent experiments have shown that some superconducting samples^{1–7} may attract magnetic field—the so-called paramagnetic Meissner effect. The scarce, if not controversial, experimental evidence for this effect makes it difficult to identify the origin of this enigmatic phenomenon, although a large number of possible explanations have been advanced^{8–16}. Here we report observations of the paramagnetic Meissner effect with a resolution better than one quantum of magnetic flux. The paramagnetic Meissner effect is found to be an oscillating function of the magnetic field (due to flux quantization) and replaces the normal Meissner effect only above a certain field when several flux quanta are frozen inside a superconductor. The paramagnetic state is found to be metastable and the Meissner state can be restored by external noise. We conclude that the paramagnetic Meissner effect is related to the surface superconductivity and, therefore, represents a general property of superconductors: on decreasing temperature, the flux captured at the third (surface) critical field inside the superconducting sheath compresses into a smaller volume, allowing extra flux to penetrate at the surface.

The evidence for the paramagnetic Meissner effect (PME) in high-temperature superconductors^{1–5} has prompted the appearance of a number of theories attributing the effect to a non-conventional superconductivity in these materials^{8–13}. Although it is possible that the proposed mechanisms do play a role in high- T_c superconductors¹⁷, more recent observations of PME in Nb (refs 6, 7) clearly indicate the existence of another, less-exotic mechanism: the limited choice of assumptions in this case makes the origin of PME more mysterious. To explain PME in terms of conventional superconductivity, theory employs the idea of flux capture inside a superconducting sample and its consequent compression with decreasing temperature^{14–16}. The flux capture can be caused by inhomogeneities^{14,15} but, in principle, could also be an intrinsic property of any finite-size superconductor due to the presence of the sample boundary¹⁶.

Here we attempt to elucidate the origin of PME by studying small (micrometre-size) superconducting disks. Confinement of superconductivity in a small volume, comparable to the characteristic superconducting lengths λ and ξ , leads to pronounced quantization, so that a mesoscopic superconductor resides in one of a series of well-resolved states, depending on temperature and magnetic field. These superconducting states are characterised by a different number and distribution of vortices^{18–21}. In comparison with the previous studies of PME on macroscopic (centimetre-size) disks, the small size of our samples gives the advantage that we can measure magnetization of individual vortex states. In addition, our samples behave very much like ideal superconductors: in the context of this work, it is important that they clearly exhibit surface superconductivity and no noticeable pinning^{18–20}. That they act in this way is probably due to their small size.

Magnetization was measured by ballistic Hall magnetometers that work as fluxmeters with a detection loop of $\sim 1 \mu\text{m}^2$ (ref. 22). The technique has a resolution of $\sim 10^4$ Bohr magnetons and is still the only technique allowing studies of individual mesoscopic superconductors below the transition temperature T_c . We have studied a number (~ 20) of superconducting disks made from Al and Nb with diameters from 0.3 to 3 μm and thicknesses t from 0.03 to 0.15 μm , using magnetometers of various widths from 1 to 2.5 μm . The behaviour described below is reproducible for identical samples and changes consistently with changing the sample parameters.

Figure 1 shows a representative set of magnetization curves measured for a 2.5- μm -diameter Al disk on cooling it down in different magnetic fields H (so-called field-cooling (FC) regime). In low fields, we observe the normal (negative) Meissner response, and below 10 G the magnetization curves are practically identical. In intermediate fields, the sign of the Meissner effect oscillates between positive and negative, depending on the particular field value, while in higher fields it stays positive, until the superconductivity is destroyed above 140 G. The detailed field dependence of the Meissner effect is shown in Fig. 2, which plots the low-temperature value of the FC magnetization (deduced from curves such as those in Fig. 1) for Al disks of diameter 1.0 and 2.5 μm . The strongly oscillating behaviour clearly seen for the larger sample is due to size quantization. Each jump corresponds to a change in the number of vortices inside the disk, which can either form an array of single-quantum vortices or assemble into a single giant vortex^{16,18–21}. The latter configuration is generally expected at fields between the second and third critical fields, $H_{c2} < H < H_{c3}$, that is, it corresponds to the surface superconductivity in a confined geometry. The smaller sample does not exhibit this rapidly oscillating field dependence, and its Meissner response remains negative over the entire field interval. Such qualitatively different behaviour is related to the fact that, in the smaller sample, the superconductivity is

suppressed by ~ 3 flux quanta, ϕ_0 , entering the disk area while $\sim 20\phi_0$ are necessary to destroy superconductivity of the larger disk^{18–20}.

To summarize the behaviour observed on other samples: we always found a diamagnetic response in low magnetic fields which gives way to a paramagnetic response only after the entry of at least several flux quanta into the disk interior, provided that the superconductivity survives to such fields (compare the two samples in Fig. 2). This seems to be in contrast to the previous studies on macroscopic samples, where the PME was normally found in very low fields and gradually disappeared with increasing field. However, one should take into account that even the lowest fields in the previous experiments allowed many thousands of flux quanta inside the sample interior. We also observe that, with decreasing disk thickness, the reversal of the sign of the Meissner effect tends to occur at lower fields and the PME magnitude becomes larger. No qualitative difference in behaviour is observed between disks of circular and square shapes.

The origin of the PME becomes evident if we compare the field dependence of the Meissner effect discussed above with the magnetization response measured by sweeping the magnetic field at a constant temperature (CT regime) (Fig. 3). Instead of a single magnetization curve characteristic of macroscopic superconductors, the spatial confinement gives rise to a family of magnetization curves corresponding to different vortex states. Several superconducting states can be realised at the same applied field (up to five as seen in Fig. 3) but only the state with the most negative CT magnetization is thermodynamically stable^{19,20}. Other states are metastable and become observable due to the presence of the surface (Bean–Livingston) barrier^{18–21}. Recent theory^{19,20} is in good agreement with the similar CT curves reported previously¹⁸.

Figure 3 clearly shows that the paramagnetic states reached via field cooling are all metastable. Indeed, the FC data predictably fall on the CT curves because only these distributions of the order

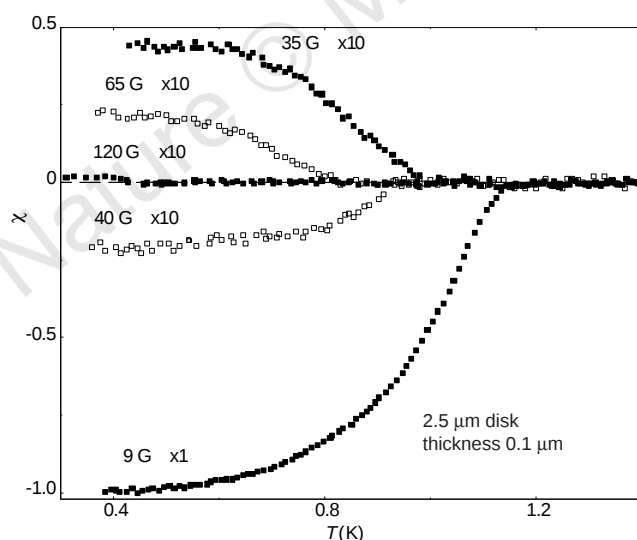


Figure 1 Magnetic susceptibility χ of an aluminium disk for various magnetic fields perpendicular to the disk surface. There is no hysteresis when sweeping the temperature up and down. Magnetization amplitude is normalized to its value at low fields (< 5 G) at 0.3 K. The top curves are multiplied by a factor of 10. At 0.3 K, the bulk critical field H_{c2} for this Al film is ~ 80 G (measured resistively); surface critical field $H_{c3} \approx 140$ G (magnetization data); $T_c \approx 1.25$ K; $\lambda(0) \approx 70$ nm; Ginzburg–Landau parameter $\kappa = \lambda\xi \approx 0.3$.

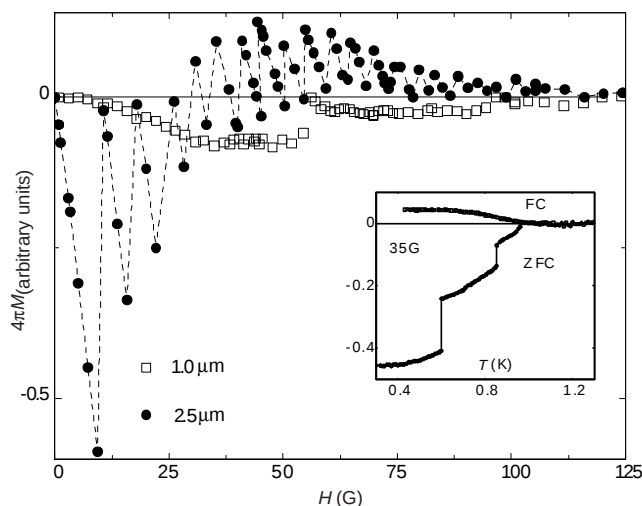


Figure 2 Detailed field dependence of the Meissner response. The figure plots the magnetic flux captured inside or expelled out of a Hall magnetometer of 2.5 μm width at 0.4 K, due to the presence of superconducting disks ($\Delta\Phi = (B) - H = 4\pi M$; ref. 22). The two disks were fabricated simultaneously by thermal evaporation and differ only in their diameters. The dashed line is a guide to the eye. The inset compares FC and zero-field-cooling (ZFC) magnetization. In the latter case, the sample is cooled down in zero field, then a field is applied and the magnetization is measured as temperature increases. The inset is for the 2.5- μm disk at the field where the paramagnetic response is close to its maximum value. The ZFC response is always diamagnetic. The jumps in the ZFC curve correspond to entry of individual vortices into the disk interior.

parameter are allowed by quantization. However, among all possible states at a given field, the system unexpectedly 'chooses' the metastable state with the most positive possible magnetization. Only if we remove the proper screening in our experimental setup does a metastable high-magnetization state eventually relax to the corresponding stable state on the lowest curve. The same result was obtained when the experiment was carried out in a more controllable manner, by applying an oscillating magnetic field at a constant H . One can verify that, according to Fig. 3, an oscillating (fluctuating) field moves the system down the ladder of curves, towards equilibrium.

How, on cooling down, the system can end up in the most thermodynamically unfavourable state may be seen from the following consideration. Superconducting states in a confined geometry can be characterised by a quantum number L corresponding to the number of nodes in the distribution of the complex order parameter Ψ along the sample circumference. For the case of a giant vortex and an array of single-quantum vortices, L has a simpler meaning: it is the angular momentum and the number of vortex cores, respectively^{18–25}. Transitions between states with different L are of first order and lead to jumps in magnetization (for example, see Fig. 3 and Fig. 2 inset)^{19,20,25}. The FC curves of Fig. 1 do not exhibit any magnetization jumps; further analysis of our experimental data shows that no jumps between different L occur at temperatures as little as 0.03 K away from the superconducting transition that on our curves corresponds to H_{C3} (refs 18–20). This proves that, on cooling down, the superconducting system preserves its L -fold symmetry as it was initially induced by the surface superconductivity at H_{C3} in the form of a giant vortex.

It is this persistence of L down to low temperatures that is responsible for the PME. We now describe a simple model, which combines the ideas of refs 14 and 16, that explains the essential physics involved. Close to H_{C3} , the magnetic field is distributed homogeneously and it requires the 'high-temperature' magnetic flux $\Phi_{HT} \approx \phi_0(L + L^{1/2})$ (ref. 24) to initiate a giant vortex with

momentum L inside a superconducting disk of radius r . As the temperature decreases below the surface superconducting transition, the superconducting sheath at the disk perimeter rapidly expands inside, compressing the giant vortex into a small volume (see Fig. 3 inset; we consider the case $\lambda \ll r$). The compressed flux inside a giant vortex is equal to $\phi_0 L$, that is, Φ_{HT} is practically conserved for $L \gg 1$. When at H_{C2} the giant vortex splits into L single-quantum vortices, the captured flux changes little²⁰. At this point, we have to take into account the fact that the magnetic field also penetrates at the disk boundary, giving rise to an additional flux through the disk of the order of $\pi r \lambda H_B$, where H_B is the field strength in the λ -layer at the surface. The magnetization response is paramagnetic if the low-temperature value of the total flux, $\Phi_{LT} \approx \phi_0 L + \pi r \lambda H_B$, is larger than Φ_{HT} . For a superconducting cylinder, $H_B = H$ and the PME appears at relatively large $L > (r/\lambda)^2$ and its amplitude is rather small ($\mu \approx \lambda/r$).

The plate geometry significantly enhances the PME because H_B is increased by demagnetization effects¹⁴. If the central region occupied by a vortex or vortices is small compared to the disk area, one can approximate $H_B \approx H(r/t)$. This yields the paramagnetic response $\mu \approx Nt$, considerable even for macroscopic thin disks. The plate geometry also leads to an earlier start of PME. This model is in good, semi-quantitative agreement with the behaviour observed in our experiment, and also explains the PME in macroscopic Nb disks^{6,7} and, possibly, in high- T_c superconductors. The latter often consist of micrometre-size grains or, owing to inhomogeneity of single crystals, can effectively mimic such a medium. $\square$

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Correspondence and requests for materials should be addressed to A.K.G. (geim@sci.kun.nl).

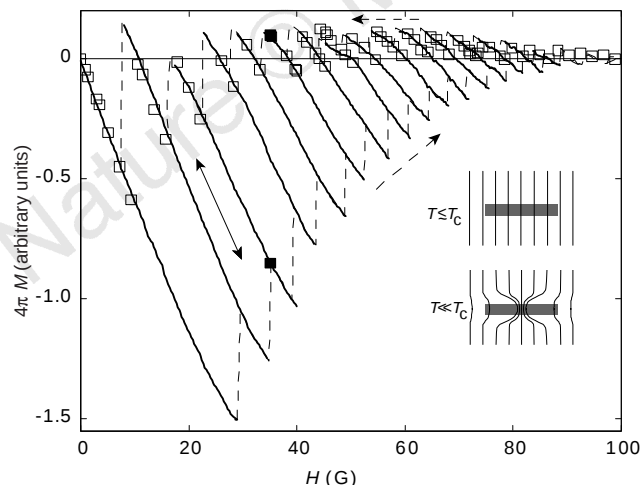


Figure 3 Comparison of the magnetization states reached by cooling in a field and by sweeping the field at a constant temperature. The field-cooled (FC) data shown by open squares are for the 2.5- μm disk of Fig. 2. The lines are for the CT regime. The solid curves were measured by pausing at various fields and then sweeping the field up and down (0.4 K). When the magnetic field is swept continuously, the magnetization evolves along one of the solid curves until it reaches the end of this curve and jumps to the next one, belonging to another vortex state. Then, the process repeats itself all over again as shown by dashed lines. Arrows show the direction of the sweep. The filled squares at 35 G indicate the low-temperature states for the FC and ZFC curves of Fig. 2. The inset illustrates the compression of a giant vortex (T close but below T_c) into a smaller volume (T further away from T_c) which allows extra flux to enter the sample at the surface.

Phonon density of states and negative thermal expansion in ZrW_2O_8

G. Ernst*, C. Broholm†‡, G. R. Kowach* & A. P. Ramirez*

* Bell Laboratories, Lucent Technologies, Murray Hill, New Jersey 07974-0636, USA

† Department of Physics and Astronomy, Johns Hopkins University, Baltimore, Maryland 21218, USA

‡ Center for Neutron Research, National Institute of Standards and Technology, Gaithersburg, Maryland 20899, USA

Thermal expansion of solids arises from anharmonic lattice dynamics. The contrasting phenomenon of negative thermal expansion (NTE)—where expansion occurs on cooling rather than heating—was discovered¹ in ZrW_2O_8 in 1968. Recently, this material has attracted interest in the context of NTE for several reasons: the magnitude of the effect is relatively large (-9 p.p.m. K^{-1}); the temperature range over which NTE occurs is also large (from close to absolute zero up to the decomposition temperature of about 1,050 K); and the NTE effect is isotropic², evidenced by the fact that ZrW_2O_8 remains cubic at all temperatures. These characteristics make ZrW_2O_8 an important system in which to study unusual lattice dynamics of this type, and potentially well suited for application in composite materials with an engineered thermal expansion coefficient³. Here we report neutron-scattering measurements of ZrW_2O_8 that allow us to investigate its phonon spectrum, and hence determine the energy scale for the lattice motions governing NTE. We find that NTE can be modelled by several low-energy phonon modes, suggesting that the effect arises from the unusual crystal structure of ZrW_2O_8 , which supports highly anharmonic vibrational modes.

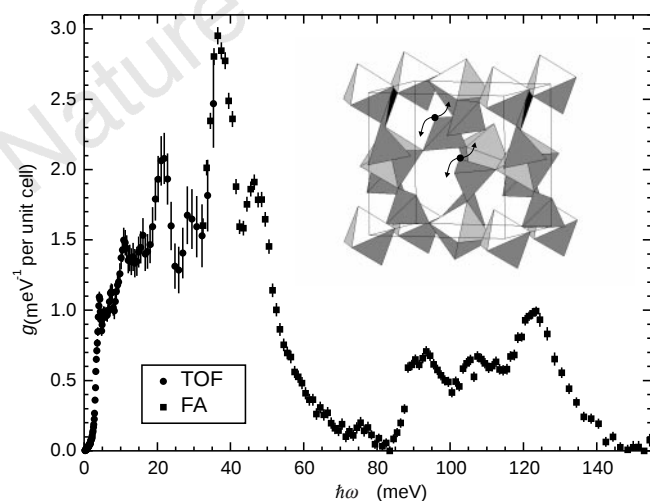


Figure 1 Generalized phonon density of states $g(\omega)$ of ZrW_2O_8 at $T = 300 \text{ K}$. Data were obtained from inelastic neutron scattering, using time-of-flight (TOF) and filter-analyser (FA) spectroscopy to probe the low and high phonon-energy range, respectively. Inset, unit cell of ZrW_2O_8 . The room-temperature crystal structure of ZrW_2O_8 is a primitive cubic Bravais lattice with 44 atoms per unit cell arranged according to space group $P2_13$. ZrW_2O_8 consists of corner-sharing WO_4 tetrahedra and ZrO_6 octahedra. Each corner of the ZrO_6 octahedra is shared with one WO_4 tetrahedron, whereas one corner of each WO_4 tetrahedra remains unshared (marked as O3 and O4 by filled circles).

Vibrational modes that lead to NTE typically involve either a transverse motion of one or more atoms or the libration of a rigid group of atoms. The process involved may be appreciated by using the 'guitar string' analogy: the transverse motion of a heavy mass suspended between two strings will tend to pull in the string supports, leading to contraction of the overall structure. The first case is realized in materials like RbI, where the volume dependence of the transverse acoustic phonons leads to NTE at low temperatures⁴. The second case is described by the rigid unit mode picture⁵ which has been applied⁶ to account for NTE in ZrW_2O_8 : in open-framework structures consisting of corner-linked polyhedra, NTE can arise when rotational modes of rigid polyhedra pull the entire structure inwards on thermal excitation. In ZrW_2O_8 , for example, one realization of a rigid unit mode which leads to NTE might involve transverse motion of the untethered oxygens, O3 and O4; in Fig. 1 inset, these oxygens are shown as filled circles, and the transverse motions are indicated by arrows. This motion will tend to pull in the neighbouring ZrO_6 octahedra, thus shrinking the crystal as a whole and leading to NTE. Examination of ZrW_2O_8 reveals similarities to the above-mentioned RbI, associated with heavy ionic moieties residing on a face-centred cubic (f.c.c.) lattice. In ZrW_2O_8 , each f.c.c. lattice site is occupied by a ZrO_6 octahedron instead of a single atom of Rb, and a pair of WO_4 tetrahedra replace each I atom. This analogy suggests that transverse acoustic modes which cause NTE in the rubidium halides also play an important role in ZrW_2O_8 .

As the lattice parameter a changes only by a few p.p.m. K^{-1} , NTE in ZrW_2O_8 can be treated as a small perturbation. For a quantitative description, the overall thermal expansion $\alpha = a^{-1} da/dT$ is usually decomposed into contributions of the fundamental vibrational modes ω_i of the crystal. The contribution α_i of each mode ω_i is given by $\alpha_i = \gamma_i c_i / 3B$, where the so-called Grüneisen parameter $\gamma_i = -\partial \ln \omega_i / \partial \ln V$ defines the relative change of the mode frequency with volume (V), c_i is the contribution of this mode to the total specific heat C_V , and B is the bulk modulus of the material. The total α is given by $\alpha = \Sigma \alpha_i = \gamma C_V / 3B$ with the overall Grüneisen parameter $\gamma = \Sigma \gamma_i c_i / \Sigma c_i$. Typically⁷, α is positive and of the order of a few p.p.m. K^{-1} and γ has a value of 2–3.

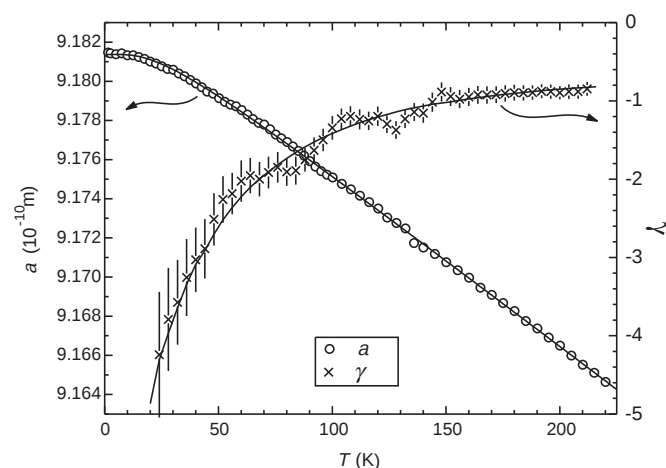


Figure 2 Lattice parameter a (open circles) and Grüneisen parameter γ (crosses) of ZrW_2O_8 as a function of temperature. These data were obtained by neutron Bragg diffraction from a single crystal of ZrW_2O_8 . To obtain $\gamma = 3\alpha B / C_V$, we use the known specific-heat data⁸. A value of $B = 4.8 \times 10^{10} \text{ N m}^{-2}$ for the bulk modulus was extracted from the measured longitudinal and transverse sound velocity of ZrW_2O_8 at room temperature. Except for deviations at the lowest temperatures, γ is found to be inversely proportional to temperature. Solid lines were calculated from $g(\omega)$ with the assumptions about the energy-dependent Grüneisen parameter shown in Fig. 3 inset.

We used inelastic neutron-scattering techniques to measure the phonon density of states $g(\omega)$ shown in Fig. 1. It consists of two groups of phonon modes, a low-energy group centred at 30 meV, and a high-energy group centred at 110 meV. We extended our measurements up to 200 meV, and found no phonon modes above 150 meV. Although the overall shape of $g(\omega)$ is typical for oxides, the energy of the lowest-energy optical phonon mode is unusually small, and significant spectral weight is found up to higher energies than is commonly seen in transition-metal oxides. The entire area under these two phonon bands should be $3 \times 44 = 132$, consisting of 3 acoustic and 129 optical modes per unit cell. The integral over the measured $g(\omega)$ reaches 96% of this value, which indicates that we have probed the entire phonon spectrum of the material.

To determine which of these modes is responsible for NTE, we performed a careful measurement of the temperature dependence of the lattice parameter a of a single crystalline sample (Fig. 2). The thermal expansion coefficient α is negative down to the lowest temperatures, and is approximately constant above 50 K with a value of $\alpha = -9.39 \pm 0.03$ p.p.m. K⁻¹. This number agrees well with previously published data² on polycrystalline ZrW₂O₈. The temperature range below 50 K, where α changes with temperature, defines an energy scale for the relevant phonons. With increasing temperature, phonons with higher energies are occupied and hence start to contribute to thermal expansion. If α is constant in a certain temperature range, phonons with the corresponding thermal energy cannot contribute significantly to the overall thermal expansion. Thus only phonons with energies below ~ 10 meV are relevant for NTE in ZrW₂O₈.

For a quantitative analysis we determined the Grüneisen parameter γ over a range of temperatures (Fig. 2). Except for deviations at the lowest temperatures, γ is found to be inversely proportional to temperature. The small oscillations around 100 K are most probably related to small systematic errors in the lattice-parameter measurements. As γ is negative and decreases on lowering the temperature, the Grüneisen parameters γ_i of the relevant low-energy phonon modes ω_i have to be negative. We find no indication of a phonon mode with a positive γ_i , which would show up as a positive contribution changing rapidly at the temperature corresponding to the energy of this mode.

These considerations lead to the following model for the energy dependence of the Grüneisen parameter in ZrW₂O₈. We isolate the

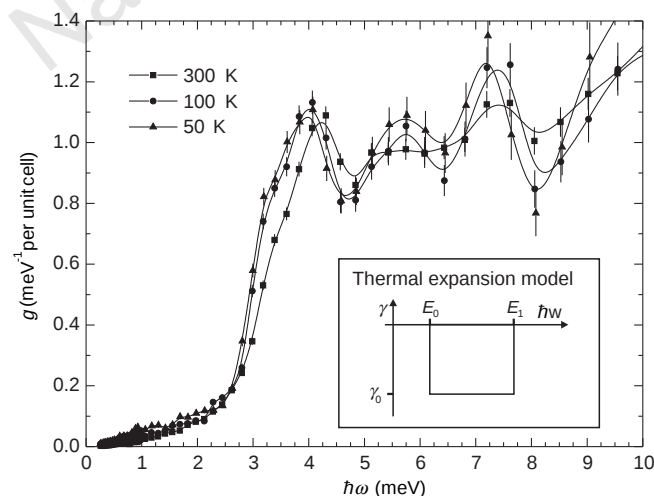


Figure 3 Low-energy generalized density of states for $T = 50$ K, 100 K and 300 K as determined from inelastic neutron scattering. Inset, the energy dependence of the Grüneisen parameter used to calculate the solid lines in Fig. 2. See text for details.

range of relevant phonon energies by assuming a lower threshold E_0 and an upper threshold E_1 such that only phonons with energies in between contribute to NTE. For these phonon modes with energies $E_0 \leq \hbar\omega \leq E_1$ we set the Grüneisen parameter constant, $\gamma_i = \gamma_0$. All other γ_i are assumed to vanish, as shown in Fig. 3 inset. With this model for γ and the measured density of states $g(\omega)$, we fit the measured $a(T)$ and then calculate the resulting $\gamma(T)$. A least-squares fit yields $\gamma_0 = -14 \pm 2$, $E_0 = 1.5 \pm 0.4$ meV, and $E_1 = 8.5 \pm 0.2$ meV, indicating that phonon modes with energies $1.5 \text{ meV} \leq \hbar\omega \leq 8.5 \text{ meV}$ are most relevant for NTE. These phonons are characterized by a relatively large and negative Grüneisen parameter of $\gamma_0 = -14$, which corresponds to a frequency shift of $\sim 3\%$ per kbar applied pressure. The solid lines in Fig. 2 show the resulting $a(T)$ and $\gamma(T)$ calculated with these parameters. We note that our model accounts well for the observed thermal expansion over a wide temperature range. Therefore, it sets the framework for more elaborate models which might attempt to define individual values of γ_i .

Figure 3 shows the part of the phonon spectrum which is relevant for NTE in ZrW₂O₈ for three different temperatures up to 300 K. Integrating over $g(\omega)$ we find five modes in the energy range up to $E_1 = 8.5$ meV. The ω^2 spectrum below $\hbar\omega = 3$ meV indicates that at least two of these five modes are acoustic. The remaining optical modes are most probably related to the motion of the heavy WO₄ tetrahedra and ZrO₆ octahedra, and hence to the class of rigid unit modes predicted⁶ for this crystal structure. However, the measured spectrum of optical modes does not go down to zero energy, as in the original rigid unit mode calculations which did not include forces between all atoms. The precise connection between the wavefunctions of the observed optical modes and the theoretical rigid unit modes remains an open question for further study.

A feature of the low-energy spectrum is the hardening of the 3.8-meV mode with increasing temperature with $(\partial\omega/\partial T)_p \approx (0.25 \text{ meV})/(250 \text{ K}) = 10^{-3} \text{ meV K}^{-1}$. This hardening leads us beyond the standard Grüneisen formalism—in which phonon frequencies depend explicitly only on volume, and not on temperature—such that the temperature dependence of the phonon frequency is directly related to its Grüneisen parameter $(\partial\omega/\partial T)_p = (\partial\omega_i/\partial V)_T (\partial V/\partial T)_p = -3\omega_i\gamma_i\alpha$. Applying this relation to the 3.8-meV mode we obtain a positive $\gamma_i \approx 8$ for this mode, in contradiction to the measured thermal expansion coefficient. Hence the hardening of the 3.8-meV mode is evidence for an explicit temperature dependence of its frequency, which could result from quartic terms in the lattice potential energy. For a quartic oscillator, the energy separation between adjacent eigenstates increases with increasing eigenstate index. Thus the average energy spacing between excited states is shifted to higher values as eigenstates are thermally occupied. Quartic terms in the ZrW₂O₈ lattice energy would in fact not be surprising, in view of the underconstrained nature of the WO₄ tetrahedra and the unusually large measured specific heat⁸ which is about 10% percent larger than calculated from $g(\omega)$ using the harmonic approximation. □

Method

Sample preparation. Single-phase ZrW₂O₈ (as determined by powder X-ray diffraction) was prepared from stoichiometric amounts of high-purity ZrO₂ and WO₃ powder. After vibratory milling, the micrometre-sized particles were uniaxially pressed into pellets of ~ 5 g each. These green pellets were heated in oxygen at 1,180 °C for 5 h and subsequently quenched to room temperature. Single crystals of ZrW₂O₈ were prepared by a non-equilibrium technique as described elsewhere (G.R.K. *et al.*, manuscript in preparation).

Elastic neutron scattering. We determined the lattice parameter by neutron Bragg diffraction from the (220) peak of a single crystal (of mass 0.8 g) on the SPINS cold neutron spectrometer at the NIST centre for neutron research. PG(004) crystals were used as monochromator and analyser with $E_i = 10.4$ meV. With a scattering angle close to 115° and with horizontal

collimations 10'-20'-20'-20' we obtained a wavevector resolution of $\delta Q/Q = 0.0020$.

Inelastic neutron scattering. A 50-g powder sample was sealed with ^4He exchange gas in a thin-walled aluminium can for the inelastic neutron-scattering experiments at the NIST centre for neutron research. For energy transfers <40 meV we used a direct geometry time-of-flight (TOF) spectrometer in the neutron energy gain mode with a PG(002) double crystal monochromator set at $E_i = 3.55$ meV and a cooled beryllium filter in the incident beam. The elastic energy resolution was 0.2 meV. Low temperature (10 K) neutron energy gain data served as a direct measure of the background. For energy transfer from 40 to 200 meV we used a filter analyser (FA) spectrometer with a Cu(220) monochromator surrounded by 60'-40' horizontal collimation and combined with a cooled polycrystalline beryllium filter as the analyser. The relative energy resolution of this instrument was $\sim 8\%$ in the energy range probed. The fast neutron background in the FA experiment was measured and subtracted. We also subtracted a constant background arising from the finite transmission through the FA of elastically scattered thermal neutrons. The scale factor between the TOF and FA data was determined by comparing the integrated intensity associated with the 37-meV peak in the phonon spectrum of ZrW_2O_8 (Fig. 1). Absolute normalization of the TOF data was accomplished by measuring the count rate associated with incoherent elastic scattering from a known amount of vanadium.

Analysis of neutron scattering data. In the limit of large wavevector transfer, Q , the one-phonon double differential scattering cross-section can be approximated^{9,10} by:

$$\frac{d^2\sigma}{d\Omega dE'} = \frac{k'}{k} N \sum_{i=1}^n \frac{(\hbar Q)^2}{2M_i} b_i^2 e^{-2W_i(Q)} Z_i(\omega) \left(\frac{\hbar(\omega) + 1}{\hbar\omega} \right) \quad (1)$$

Here the sum is over all atoms in one unit cell, k and k' are the initial and final neutron wavevectors, respectively, b_i is bound coherent neutron scattering length, M_i is the atomic mass, $Z_i(\omega)$ is the contribution of atom i to the phonon density of states and $n(\omega) = (\exp(\hbar\omega/k_B T) - 1)^{-1}$ is the Bose population factor. The Debye-Waller factor, $\exp(-2W_i(Q))$, is related to the mean-square atomic displacements given by Evans *et al.*¹¹ The quantity which can be extracted from inelastic neutron scattering is the so-called generalized phonon density of states $g(\omega)$ defined by:

$$g(\omega) = \sum_{i=1}^n \frac{b_i^2}{2M_i} e^{-2W_i(Q)} Z_i(\omega) / \left(\sum_{i=1}^n \frac{b_i^2}{2M_i} e^{-2W_i(Q)} \right) \quad (2)$$

Concerning the relationship between $g(\omega)$ and $Z(\omega)$ we note that for ZrW_2O_8 , the neutron scattering lengths of the different atoms are very similar, and the differences in the atomic masses are partially compensated by the Debye-Waller factors. Nevertheless, we expect that vibrations of the lighter oxygen atoms are weighted more heavily in $g(\omega)$ than in the true state density, $Z(\omega)$.

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Correspondence and requests for materials should be addressed to A.P.R. (e-mail: apr@bell-labs.com).

Five parametric resonances in a microelectromechanical system

Kimberly L. Turner*, Scott A. Miller†‡, Peter G. Hartwell§, Noel C. MacDonald§, Steven H. Strogatz* & Scott G. Adams*†

* Department of Theoretical and Applied Mechanics, † School of Applied and Engineering Physics, § School of Electrical Engineering and the Cornell Nanofabrication Facility, Cornell University, Ithaca, New York 14853-5401, USA
‡ Present address: Kionix, Inc., 22 Thornwood Drive, Ithaca, New York 14850, USA.

The Mathieu equation¹ governs the forced motion of a swing², the stability of ships³ and columns⁴, Faraday surface wave patterns on water^{5,6}, the dynamics of electrons in Penning traps⁷, and the behaviour of parametric amplifiers based on electronic⁸ or superconducting devices⁹. Theory predicts that parametric resonances occur near drive frequencies of $2\omega_0/n$, where ω_0 is the system's natural frequency and n is an integer ≥ 1 . But in macroscopic systems, only the first instability region can typically be observed, because of damping and the exponential narrowing¹⁰ of the regions with increasing n . Here we report parametrically excited torsional oscillations in a single-crystal silicon microelectromechanical system. Five instability regions can be measured, due to the low damping, stability and precise frequency control achievable in this system. The centre frequencies of the instability regions agree with theoretical predictions. We propose an application that uses parametric excitation to reduce the parasitic signal in capacitive sensing with microelectromechanical systems. Our results suggest that microelectromechanical systems can provide a unique testing ground for dynamical phenomena that are difficult to detect in macroscopic systems.

The micromachining field has been given the generic name microelectromechanical systems. This field is quite broad, and

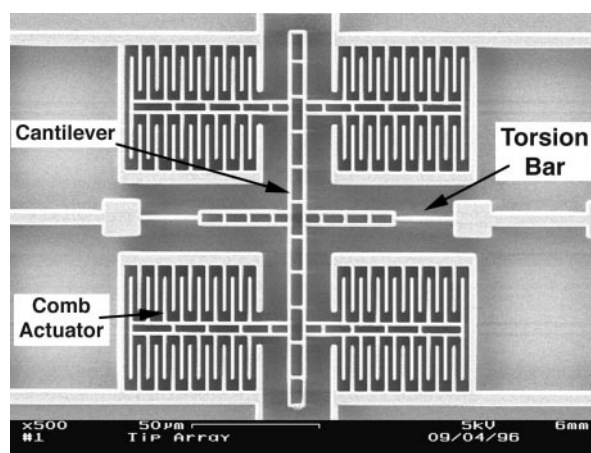


Figure 1 Scanning electron microscope image of the torsional oscillator. This oscillator is the out-of-plane motion actuator for arrays of scanning tunnelling microscopes¹². A typical device covers an area of $\sim 150 \mu\text{m}^2$. The relevant parameters for this device are: $k \approx 2.75 \times 10^{-8} \text{ N m}$, $I = 2.12 \times 10^{-19} \text{ kg m}^2$, $\gamma = 1.216 \times 10^{-12} \text{ N m V}^{-2}$ and $Q \approx 3000$ (see text for definition of symbols). The maximum signal amplitude applied to the device was 38 V. The device is fabricated using conventional integrated-circuit technology from single-crystal silicon.

includes integrated microsensors, microactuators, microinstruments¹¹, micro-optics and microfluidics. Applications include accelerometers to deploy an air bag in a car; ink-jet printer heads; an array of movable mirrors for colour projection displays; and atom probes for imaging and transporting atoms^{12,13}. Microinstrumentation for micrometre/nanometre scale fluidics¹⁴, when coupled with microanalytical instrumentation, offers possibilities in characterizing mechanical and rheological properties of biological molecules. Microelectromechanical systems typically use silicon as a structural material, and the devices are fabricated using conventional integrated-circuit technology.

The devices we consider here are microelectromechanical probes used for scanned-probe microscopy, including scanning tunnelling

microscopy and atomic force microscopy. They are fabricated from single-crystal silicon. As shown in Fig. 1, the device consists of a cantilevered beam connected to a torsion bar. Attached to the cantilevered beam are an atomically sharp tip for the probe and comb capacitive transducers for sensing and actuation. The same capacitive transducer design, which is the source of the parametric excitation in this system, can be used either for sensing displacements or for inducing motion. As an actuator, the interdigitated capacitive plates used here allow for a wide range of motion and reasonably high amplitudes without failure. This type of actuator generates out-of-plane motion forces due to a phenomenon known as comb-drive levitation¹⁵. In this technique, a voltage is applied to the fixed electrodes, while the silicon substrate and movable electrodes are grounded. This causes asymmetrical fringing electric fields between the movable and fixed electrodes, and these fields induce motion in the movable electrodes, one of which is illustrated in a cross-sectional view in Fig. 2a.

To estimate the out-of-plane forces and torques generated by the comb-drive levitation effect, we have performed a three-dimensional electrostatic simulation using COULOMB¹⁶, an electrostatic boundary element method solver. A graph of the computed electrostatic torque versus angle of rotation for one movable electrode is shown in Fig. 2b.

The operational mode of the device is an out-of-plane torsional vibration. Although the system is continuous, much of its behaviour can be understood by a simplified one-degree-of-freedom model that treats the torsional mode separately. The equation of motion is:

$$I\ddot{\theta} + c\dot{\theta} + k\theta = M(t, \theta) \quad (1)$$

where θ is the rotation angle of the torsion bar (the overdot denotes differentiation with respect to time), I is the mass moment of inertia of the torsional cantilever, c is the torsional damping constant, k is the torsional stiffness, and M is the applied torque. As a first approximation, we assume that the damping and restoring forces are linear. For the torsional members used here, made of single-crystal silicon, the quality factor Q has been measured to be $\sim 3,000$ at 18 mtorr. The torque is $M(t, \theta) = V^2\phi(\theta)$, where V is the applied voltage and $\phi(\theta)$ gives the angular dependence of the torque generated by comb-drive levitation. Applying a voltage $V = (A_{DC} + A_{AC}\cos\omega t)^{1/2}$ yields $M(t, \theta) = (A_{DC} + A_{AC}\cos\omega t)\phi(\theta)$, where A_{DC} and A_{AC} are the magnitudes of the direct current and alternating current of the input signal, respectively. From results of the simulations shown in Fig. 2b, $\phi(\theta)$ can be approximated as linear

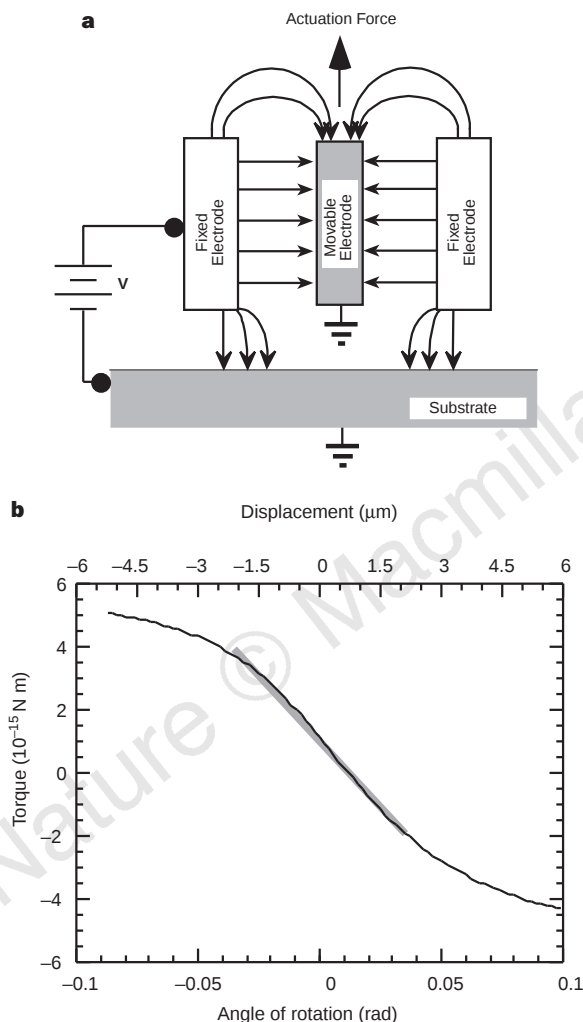


Figure 2 Comb-drive levitation of torsional oscillator. **a**, Schematic of comb-drive levitation with one movable electrode and two fixed electrodes. The actual device has a total of 16 movable electrodes on each side. In modelling, we assumed that the metal-coated interdigitated fingers were perfect conductors. The beams were modelled as 1 μm wide, 20 μm long and 10 μm deep, with a 2 μm gap between electrodes and a distance of 5 μm between the device electrodes and the substrate. The simplified model consisted of one movable electrode between two fixed ones. The results were then generalized to include all 32 actuation electrodes in the particular device being studied. **b**, Torsional simulation results: torque generated versus angle of rotation for one movable electrode and two fixed electrodes. Displacement of the end of the cantilever is also displayed on the top axis. During device operation, the maximum rotation obtained is 0.017 rad. This shows that for the region of operation we are concerned with, the torque generated versus angle rotated curve can be approximated as linear ($R = 0.997$). The region of possible operation on this curve is highlighted, to show that the linear approximation is suitable in this case.

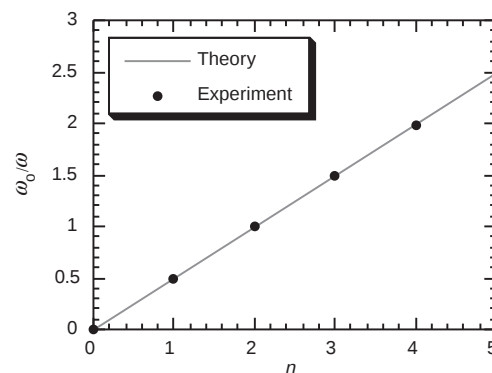


Figure 3 Theoretical prediction and experimental measurements of the frequency ratio for the centre of the n th parametric resonance region. Results for five regions are shown. The instability frequencies match theoretical values to within 0.7%. Reasons for error between predicted values and actual instabilities include errors in the calculated device parameters, errors in the force simulation (geometrical assumptions) and in the ability to accurately locate the 'centre' of the instability region, as the regions tend to be asymmetric due to damping effects.

if we assume small-angle rotations: $\phi(\theta) = -\gamma\theta$. In actual operation, the device rotates a maximum of 0.017 rad, making the small-angle approximation appropriate. Using this approximation and defining $\tau = \omega t$, the equation of motion becomes:

$$\theta'' + \frac{c}{\omega I} \theta' + \frac{1}{\omega^2 I} (k + \gamma A_{DC} + \gamma A_{AC} \cos \tau) \theta = 0 \quad (2)$$

where prime denotes differentiation with respect to τ .

Equation (2) is a damped Mathieu equation of the form

$$\theta'' + a\theta' + (b + d\cos\tau)\theta = 0 \quad (3)$$

where $a = c/\omega I$, $b = (k + \gamma A_{DC})/\omega^2 I$, and $d = \gamma A_{AC}/\omega^2 I$. The theory developed for the Mathieu equation allows us to predict the frequencies where parametric resonances will occur. Assuming weak periodic forcing and negligible damping, first-order perturbation theory predicts instabilities centred at $b = n^2/4$, for $n = 1, 2, \dots$. This result implies that parametric resonance occurs at drive frequencies $\omega = 2\omega_0/n$, where ω_0 denotes the natural frequency of the torsional mode.

To test these predictions experimentally, we used an unusual technique in which a laser vibrometer is mounted on an optical

microscope. The laser beam is sent through the microscope and focused on the movable microelectromechanical device. This enables nanometre-scale displacements of the device to be accurately resolved. After a preliminary estimate of the frequency of the first Mathieu instability, given by $\omega = 2\omega_0 = 2\sqrt{k/I}$, an experimental frequency sweep was used to establish ω_0 more precisely. Then the formula $\omega = 2\omega_0/n$ predicted the drive frequencies where the other Mathieu instabilities should be centred.

Five Mathieu instabilities predicted by equation (3) were experimentally observed. Figure 3 plots the frequency ratio ω_0/ω for the observed Mathieu instabilities, along with the theoretical prediction $\omega_0/\omega = n/2$. The agreement is within 0.7% at all points.

The theory of the Mathieu equation shows that each resonance occurs within a 'tongue' in the (b, d) parameter space for equation (3). The shape and position of the tongues are affected by damping of the system. Damping has the stabilizing effect of 'raising' the tongue higher up in the parameter space, as well as narrowing the unstable regions. Because of the extreme narrowness of high-order tongues, they are hard to resolve and are rarely observed in macroscopic systems. In our system, several Mathieu tongues could be mapped experimentally, due to the device's low damping, the precise and stable frequency control on the drive, and the sensitivity of the laser vibrometer technique. Figure 4 is a plot of the instability regions for $n = 1$ and $n = 3$. Upper limits in d for each instability are not absolute; they are merely where each experiment was stopped in order to limit the voltage amplitude to the device. The measured boundaries between stability and instability are linear for $n = 1$, which is predicted for the damped Mathieu equation.

The parametric resonances reported here suggest a way to reduce the parasitic signals in capacitive sensing with microelectromechanical systems. The motion of many such systems can be detected as changes in the capacitance between movable and fixed elements. Unfortunately, parasitic signals can be a problem; because the devices are small, and the electrical isolation is not perfect, there is some coupling of the actuating drive signal to the sensing capacitors. This stray signal can easily overshadow any change in capacitance due to the motion of the system in question.

The idea is to separate the drive and sense signals by parametrically exciting the device far from its natural frequency. This is only possible in systems governed by Mathieu-type equations, due to the unique way energy is transferred during parametric excitation. For example, we consider a given device with a natural frequency of $\omega_0 = 57$ kHz driven at the frequency ω corresponding to the first Mathieu instability ($n = 1$); here $\omega = 2\omega_0/n = 114$ kHz. The parasitic signal from the incoming drive will also be at 114 kHz. However, the device will still vibrate torsionally at its natural frequency of 57 kHz, so the capacitive sensing signals of interest will also be at 57 kHz. With this separation in frequency, it is straightforward to filter out the parasitic 114-kHz signal, thereby revealing the 57-kHz sensing signal.

Additionally, when operated in the first instability region, the device can be used to increase sensitivity in atomic force microscopy (AFM) measurements. In conventional AFM, high Q leads to higher sensitivity, at the expense of bandwidth. By utilizing the parametric resonance instability, the effect of Q can be decoupled from sensitivity. At the edge of the instability region, the device has a 0.001 Hz in 114 kHz turn-on (that is, a shift of 10 parts per billion will move the response from stable to unstable). This is a much sharper transition than obtainable with high- Q cantilevers used in present resonance mode AFM techniques. □

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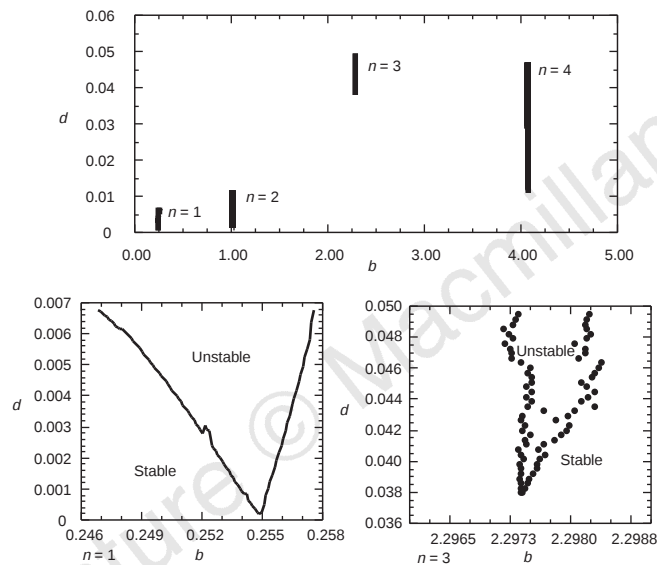


Figure 4 Instability map for $n = 1$ –4. The top panel shows a general view of this map. When studying parametric resonance in such systems, it is useful to consider only those instabilities that correspond to odd n . When using comb-drive levitation for actuation, there is a small asymmetry in the torque versus angle function (see Fig. 2b). This asymmetry directly adds an additional driving term to the right-hand side of equation (1). For driving frequencies which are not integer multiples of the natural frequency of the device, the term will not affect the Mathieu response of the system. However, when the natural frequency is an integer multiple of the drive frequency (that is, $n = 2, 4$) the driving term on the right-hand side will have fundamental and/or harmonic components which are at the natural frequency of the device, therefore driving it into resonance and coupling with any parametric resonance also present. As the first and third regions can be directly compared to theory, experimental plots of these regions are magnified to show more detail (bottom two panels). Of considerable interest is the linear shape of the $n = 1$ boundary as predicted from theory. The bottom of the curve rounds off due to damping. The boundaries for the third instability region are not linear. This is also predicted from theory¹. Differences between the mathematical prediction of the shape and the actual shape of the region are primarily due to imperfect stability of the testing set-up. Localized laser heating, drift of the laser spot on the device, room conditions, and variable conditions in the vacuum chamber also contribute to small shifts in the instability boundaries. For higher-order stability maps ($n \geq 3$), the tests can take up to 36 hours to run. Stability over time becomes an issue, as small changes in the system cause the instability boundary to shift.

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Correspondence and requests for materials should be addressed to K.L.T. (e-mail: turner@tam.cornell.edu).

Generalized syntheses of large-pore mesoporous metal oxides with semicrystalline frameworks

Peidong Yang*, Dongyuan Zhao*†, David I. Margolese*, Bradley F. Chmelka*‡ & Galen D. Stucky*‡

* Departments of Chemistry and Materials, † Materials Research Laboratory, ‡ Department of Chemical Engineering, University of California, Santa Barbara, California 93106, USA

Surfactants have been shown to organize silica into a variety of mesoporous forms, through the mediation of electrostatic, hydrogen-bonding, covalent and van der Waals interactions^{1–8}. This approach to mesostructured materials has been extended, with sporadic success, to non-silica oxides^{5–17}, which might promise applications involving electron transfer or magnetic interactions. Here we report a simple and versatile procedure for the synthesis of thermally stable, ordered, large-pore (up to 140 Å) mesoporous metal oxides, including TiO₂, ZrO₂, Al₂O₃, Nb₂O₅, Ta₂O₅, WO₃, HfO₂, SnO₂, and mixed oxides SiAlO_{3.5}, SiTiO₄, ZrTiO₄, Al₂TiO₅ and ZrW₂O₈. We used amphiphilic poly(alkylene oxide) block copolymers as structure-directing agents in non-aqueous solutions for organizing the network-forming metal-oxide species, for which inorganic salts serve as precursors. Whereas the pore walls of surfactant-templated mesoporous silica¹ are amorphous, our mesoporous oxides contain nanocrystalline domains within relatively thick amorphous walls. We believe that these materials are formed through a mechanism that combines block copolymer self-assembly with complexation of the inorganic species.

In a typical synthesis, 1 g of poly(alkylene oxide) block copolymer HO(CH₂CH₂O)₂₀(CH₂CH(CH₃)O)₇₀(CH₂CH₂O)₂₀H (designated EO₂₀PO₇₀EO₂₀; Pluronic P-123, BASF) was dissolved in 10 g of ethanol (EtOH). To this solution, 0.01 mol of the respective inorganic chloride precursor (Table 1) was added with vigorous stirring for 1/2 h. The resulting sol solution was gelled in an open Petri dish at 40 °C in air for 1–7 days, during which the inorganic precursor hydrolyses and polymerizes into a metal oxide network. Alternatively, the sol solution can be used to prepare thin films by dip coating. All as-made samples are transparent except for the samples derived from WO₃, which are dark blue. The as-made bulk samples or thin films were then calcined at 400 °C for 5 h in air to remove the surfactant species. Figure 1a shows typical X-ray diffraction (XRD) patterns for mesostructured zirconium oxides before and after calcination. The as-made zirconium inorganic/polymer mesostruc-

ture shows three diffraction peaks with lattice spacings $d = 115$, 65 and 59 Å. After calcination, the diffraction peaks appear at higher 2θ angles with $d = 106$, 60 and 53 Å. Both sets of diffraction peaks can be indexed as the (100), (110) and (200) reflections from two-dimensional hexagonal mesostructures with lattice constants $a = 132$ and 122 Å, respectively.

When HO(CH₂CH₂O)₁₀₆(CH₂CH(CH₃)O)₇₀(CH₂CH₂O)₁₀₆H (abbreviated as EO₁₀₆PO₇₀EO₁₀₆) and HO(CH₂CH₂O)₇₅(CH₂CH(CH₃)O)₄₅H (that is, EO₇₅BO₄₅) are used as the structure-directing agents under the above conditions, cubic mesophases are formed. Figure 1b shows the XRD patterns for the mesostructured TiO₂ formed using EO₇₅BO₄₅. The low-angle XRD pattern for the as-made samples shows six resolved peaks with d spacings of 100, 70, 58, 44, 41 and 25 Å. These peaks display d -value ratios of $\sqrt{2} : \sqrt{4} : \sqrt{6} : \sqrt{10} : \sqrt{12} : \sqrt{32}$, which are indexable as (110), (200), (211), (310), (222) and (440) reflections respectively, in the cubic $Im\bar{3}m$ space group. Following calcination of the sample at 400 °C, three XRD peaks are observed with d spacings of 76, 53 and 43 Å (ratios $\sqrt{2} : \sqrt{4} : \sqrt{6}$), which are similarly indexable as (110), (200) and (211) reflections of the cubic $Im\bar{3}m$ mesophase.

This synthetic procedure has been successfully applied to the preparation of mesoporous TiO₂, ZrO₂, Al₂O₃, Nb₂O₅, WO₃, HfO₂, SnO₂, and mixed oxides SiAlO_{3.5}, SiTiO₄, ZrTiO₄, Al₂TiO₅, ZrW₂O₈. Table 1 summarizes the d_{100} values measured for these different calcined oxides, along with other physical characteristics and material properties. All calcined samples show ordering lengths that are greater than 70 Å.

The appearance of low-angle diffraction peaks indicates that mesoscopic order is preserved in the calcined metal oxide materials. This is confirmed by transmission electron microscopy (TEM) images obtained from each of these samples. For example, Fig. 2a–f shows TEM images of mesoporous TiO₂, ZrO₂, Nb₂O₅ and SiAlO_{3.5} recorded along the [110] and [001] zone axes of the mesostructures. In each case, well-ordered large channels are clearly observed to be arranged in hexagonal arrays. The pore/channel walls are continuous and have thicknesses of ~40–70 Å (Table 1). In addition, energy dispersive X-ray spectroscopy (EDX) measurements and quantitative elemental analysis made on each of the calcined samples show the expected primary metal element signals, along with very weak Cl signals, which confirm that the inorganic walls consist of predominantly metal–oxygen networks. Local EDX

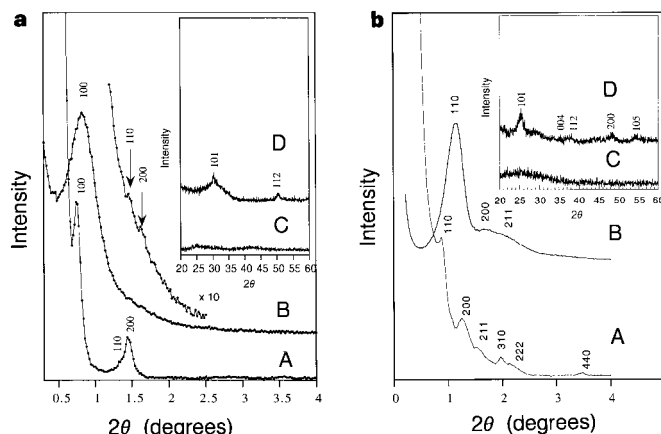


Figure 1 Powder X-ray diffraction patterns for mesoporous ZrO₂ (a) and TiO₂ (b). Traces A and C are low-angle and wide-angle XRD patterns of the as-made inorganic/EO₂₀PO₇₀EO₂₀ composite mesostructures, which have amorphous frameworks. Traces B and D are low-angle and wide-angle XRD patterns for the same mesoporous oxides calcined at 400 °C for 5 h in air. The high-angle diffractions are indexed according to their corresponding crystalline oxide phases. The XRD patterns were obtained with a Scintag PADX diffractometer using Cu K α radiation.

composition analyses on mesoporous mixed oxides indicate that the different elements are distributed uniformly within the inorganic framework.

One advantage of the thick inorganic oxide walls is that domains of nanocrystallinity can be produced in the frameworks, while

preserving the mesostructural integrities of the materials. For mesostructured materials with thinner framework walls, for example the M41S family of mesoporous solids, crystallization of inorganic species during the cooperative inorganic/organic assembly generally leads to macroscopic phase separation of the inorganic

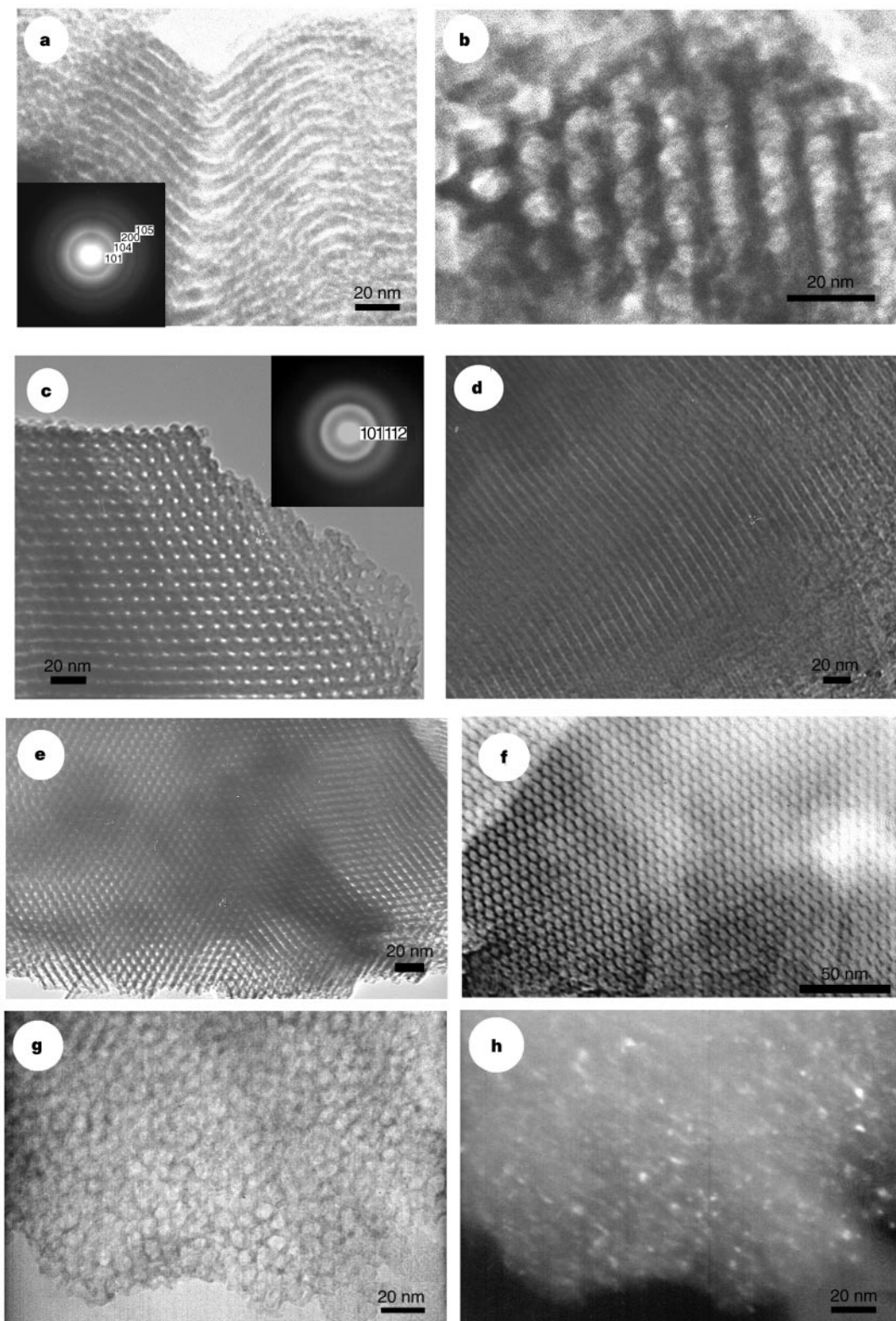


Figure 2 TEM images of mesoporous materials. Shown are TEM micrographs of two-dimensional hexagonal mesoporous TiO_2 (**a**, **b**), ZrO_2 (**c**, **d**), Nb_2O_5 (**e**) and $\text{SiAlO}_{3.5}$ (**f**). **a**, **d** are recorded along the $[110]$ zone axis and **b**, **c**, **e**, **f** along the $[001]$ zone axis, respectively, of each material. Insets in **a** and **c** are selected-area electron diffraction patterns obtained on the image area. **g**, Bright-field TEM

image of a thin slice of the mesoporous TiO_2 sample. **h**, Dark-field image obtained on the same area of the same TiO_2 sample. The bright spots in the image correspond to TiO_2 nanocrystals. The images were recorded with a 200 kV JEOL-2000 TEM. All samples were calcined at 400°C for 5 h in air to remove the block copolymer surfactant species.

Table 1 Physicochemical properties of mesoporous metal oxides

Oxide	Inorganic precursor	d_{100}^* (Å)	Wall structure	Wall thickness† (Å)	Nanocrystal size‡ (Å)	Pore size (Å)	BET surface area ($\text{m}^2 \text{g}^{-1}$)	BET surface area ($\text{m}^2 \text{cm}^{-3}$)	Porosity§	Physical properties
ZrO ₂	ZrCl ₄	106	Tetra. ZrO ₂	65	15	58	150	884	0.43	Dielectric
TiO ₂	TiCl ₄	101	Anatase	51	24	65	205	867	0.46	Semiconductor
Nb ₂ O ₅	NbCl ₅	80	Nb ₂ O ₅	40	<10	65	196	876	0.50	Dielectric
Ta ₂ O ₅	TaCl ₅	70	Ta ₂ O ₅	40	<10	35	165	1,353	0.50	Dielectric
WO ₃	WCl ₆	95	WO ₃	50	20	50	125	895	0.48	Semiconductor
SnO ₂	SnCl ₄	106	Cassiterite	50	30	68	180	1,251	0.52	Semiconductor
										$E_g = 4.05 \text{ eV}^\parallel$
HfO ₂	HfCl ₄	105	Amorphous	50	–	70	105	1,016	0.52	Dielectric
Al ₂ O ₃	AlCl ₃	186	Amorphous	35	–	140	300	1,188	0.61	Dielectric
SiO ₂	SiCl ₄	198	Amorphous	86	–	120	810	1,782	0.63	Dielectric
SiAlO _{3.5}	SiCl ₄ /AlCl ₃	95	Amorphous	38	–	60	310	986	0.59	Dielectric
Si ₂ AlO _{5.5}	SiCl ₄ /AlCl ₃	124	Amorphous	40	–	100	330	965	0.55	Dielectric
SiTiO ₄	SiCl ₄ /TiCl ₄	95	Amorphous	50	–	50	495	1,638	0.63	Dielectric
Al ₂ TiO ₅	AlCl ₃ /TiCl ₄	105	Amorphous	40	–	80	270	1,093	0.59	Dielectric
ZrTiO ₄	ZrCl ₄ /TiCl ₄	103	Amorphous	35	–	80	130	670	0.46	Dielectric
ZrW ₂ O ₈	ZrCl ₄ /TiCl ₄	100	Amorphous	45	–	50	170	1,144	0.51	NTE#

All samples were prepared using ethanol as a solvent, except HfO₂ where butanol was used. The structure-directing agent in all cases was EO₂₀PO₇₀EO₂₀ (see text). The aging process generally took 1–7 days.

* d -values of (100) reflection for samples calcined at 400 °C for 5 h in air.

† Thicknesses measured from TEM experiments. These values are consistent with the values estimated by subtracting the pore diameter from $2d_{100}/\sqrt{3}$.

‡ Nanocrystal sizes estimated from X-ray diffraction broadening using the Scherrer formula.

§ The porosity is estimated from the pore volume determined using the adsorption branch of the N₂ isotherm curve at the $P/P_0 = 0.983$ single point.

|| Nucleation just started, extremely broad wide-angle diffraction.

¶ Direct allowed optical gap estimated from $(\alpha h\nu)^2 - h\nu$ plot of UV-vis absorption measurement, where α is the absorption coefficient.

Materials with negative thermal expansion properties.

and organic components. This is because crystallization energies often dominate the interaction energies that stabilize the inorganic–organic interface, thereby disrupting the establishment of curved surfaces with three-dimensional mesostructural order^{18,19}. This is particularly the case for non-lamellar phases^{19,20}. In our approach, initial self-assembly of mesoscopically ordered as-synthesized materials with thick amorphous inorganic walls (no high-angle diffractions) provides a starting point from which nanocrystalline domains within the walls can nucleate. Relatively high densities of nanocrystallites can be produced within the thick framework walls, whose partially crystalline structure effectively sustains the local strain caused by the crystallization and prevents the mesostructure from collapsing.

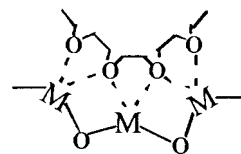
X-ray diffraction studies on samples calcined at temperatures between 100 and 400 °C indicate that this crystallographic nucleation actually occurs during the calcination, but is limited to formation of nanocrystallite domains. Wide-angle X-ray diffraction studies of calcined mesoporous TiO₂, SnO₂, ZrO₂ and WO₃ samples clearly show broad peaks that can be indexed according to their corresponding crystalline oxide phases. For example, the traces ‘D’ in Fig. 1a and b insets show wide-angle diffraction patterns for the calcined ZrO₂ and TiO₂ samples. These data indicate that the inorganic framework may consist of nanocrystalline oxide domains. However, on the basis of this evidence alone, the possibility that these nanocrystallites are associated with phase separation cannot be strictly excluded^{19,21}.

An important technique to resolve this question and of particular value for the characterization of mesoscopically ordered semicrystalline inorganic frameworks is bright-field/dark-field TEM imaging, which has not previously^{9,21} been used to substantiate claims of wall crystallinity in mesoporous materials. Selected-area electron diffraction patterns recorded on mesoporous TiO₂, SnO₂, ZrO₂ and WO₃ confirm that the walls of our materials are made up of nanocrystalline oxides that show characteristic diffuse electron diffraction rings (Fig. 2a and c insets). Figure 2g, h shows bright-field and dark-field TEM images recorded on the same area of a thin mesoporous TiO₂ sample. As can be seen in the dark-field image (Fig. 2h), the TiO₂ nanocrystals (~30 Å) are uniformly embedded in the continuous amorphous inorganic matrix to form semicrystalline wall structures. The nanocrystallite sizes are consistent with those estimated using the Scherrer formula from X-ray diffraction peak broadening (~10–30 Å, Table 1).

Calcination thus yields thermally stable ordered mesoporous solids with nanocrystalline domains in the inorganic oxide frame-

works. N₂ adsorption/desorption isotherms showing similar type-IV curves²² and porosities (~50%, Table 1) are obtained for the different calcined mesoporous oxides. Figure 3 shows N₂ adsorption and desorption isotherms that are representative of mesoporous TiO₂ and WO₃. Barrett–Joyner–Halenda (BJH) analyses show that the calcined TiO₂ and WO₃ exhibit pore sizes of 65 and 50 Å (Fig. 3 insets), respectively. The pore sizes (50–140 Å) of the mesoporous metal oxides listed in Table 1 are substantially larger than any of the non-silica mesoporous oxides previously reported^{9–12}. Large hysteresis loops with shapes that are intermediate between typical H₁- and H₂-type²² isotherms are observed for these mesoporous metal oxides. Such strong hysteresis is believed to be related to the capillary condensation associated with large pore channels²² but may also be due to modulation of the channel structure.

Mesostructured inorganic/block copolymer composites for oxides of Ga, Ge, V, Zn, Cd, In, Sb, Mo, Re, Ru, Fe, Ni, Cr, Mn and Cu have also been synthesized using this block-copolymer/inorganic-salt methodology. The formation of large-pore mesoscopically ordered metal oxides with such simplicity and generality suggests that the same inorganic-oxide/block-copolymer assembly mechanisms may be operating. In fact, it is well-documented that alkylene oxide segments can form crown-ether-type complexes with many inorganic ions through weak coordination bonds²³. After hydrolysis, the multivalent metal species (M) can associate preferentially with the hydrophilic poly(ethylene oxide) (PEO) moieties mediated by HCl, as indicated below.



The resulting complexes then self-assemble according to the mesoscopic ordering directed principally by the microphase separation of the block copolymer species. Subsequent cross-linking and polymerization of the inorganic species occurs to form the mesoscopically ordered inorganic/block-copolymer composites. The use of the different relative hydrophobicities of PEO and poly(propylene oxide) has similarly been used to assemble mesostructured SiO₂ in an aqueous solution^{8,24,25}. The proposed assembly mechanism for these diverse mesoporous metal oxides uses PEO–metal complexation interactions, in conjunction with (for example) electrostatic, hydrogen bonding, and van der Waals forces to direct mesostructure formation^{1–5}.

In addition, carrying out the assembly process in non-aqueous

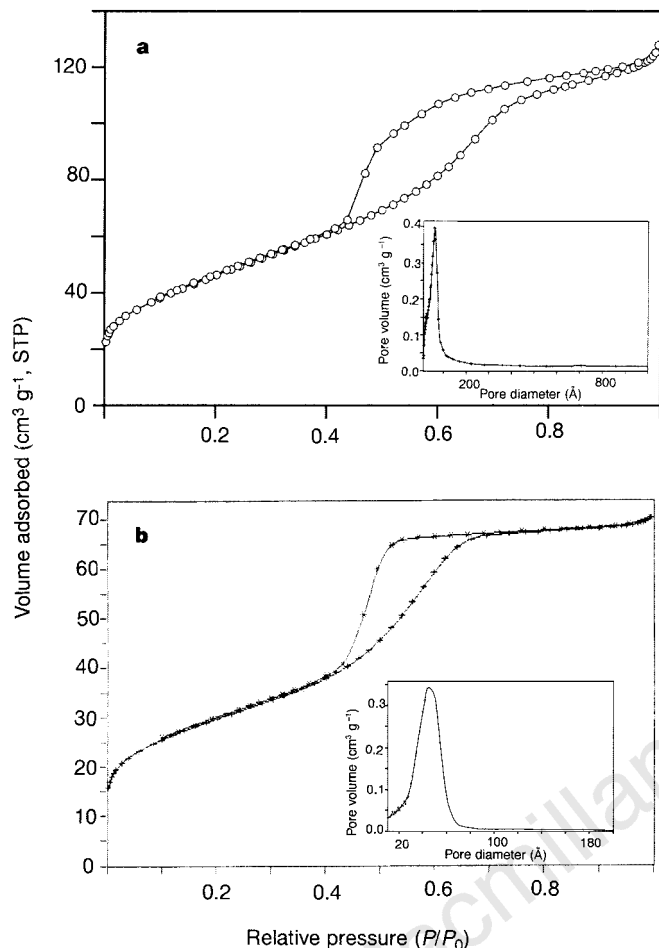


Figure 3 Nitrogen adsorption-desorption isotherms and pore-size distribution plots (insets) for calcined TiO_2 (a) and calcined WO_3 (b). Data were calculated using the BJH model from the adsorption branch isotherm. Both TiO_2 and WO_3 samples were calcined at 400°C for 5 h in air. The isotherms were measured using a Micromeritics ASAP 2000 system. The samples were outgassed overnight at 200°C before the analyses.

media using metal halides as the inorganic precursors effectively slows the hydrolysis/condensation rates of the metal species and hinders subsequent crystallization²⁶. Restrained hydrolysis and condensation of the inorganic species appears to be important for forming mesophases of most of the non-silica oxides, because of their strong tendency to precipitate and crystallize into bulk oxide phases directly in aqueous media. Previous work on metal oxide mesostructure formation has been mostly carried out under aqueous conditions using reactive alkoxides as the inorganic precursors. Special processing, including for example the addition of stabilizing agents, is needed to slow the hydrolysis and condensation rates as demonstrated for TiO_2 (ref. 11). We have adopted a procedure that can regulate the hydrolysis/condensation and self-assembly processes simultaneously. We anticipate that with this method, the syntheses of other mesoscopically ordered inorganic compounds (including sulphides¹⁶) should also be possible. Such mesoporous materials with increasing richness of inorganic compositions have excellent potential for applications as high-surface-area battery electrodes, sensors, optoelectronic devices and catalysts. □

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Correspondence and requests for materials should be addressed to G.D.S. (e-mail: stucky@chem.ucsb.edu).

Low interannual variability in recent oceanic uptake of atmospheric carbon dioxide

Kitack Lee*†, Rik Wanninkhof†, Taro Takahashi‡, Scott C. Doney§ & Richard A. Feely||

* Rosenstiel School of Marine and Atmospheric Science/CIMAS, University of Miami, 4600 Rickenbacker Causeway, Miami, Florida 33149, USA

† Atlantic Oceanographic and Meteorological Laboratory, NOAA, Miami, Florida 33149, USA

‡ Lamont-Doherty Earth Observatory of Columbia University, Palisades, New York 10964, USA

§ Climate and Global Dynamics, NCAR, Box 3000, Boulder, Colorado 80307, USA

|| Pacific Marine Environmental Laboratory, NOAA, Seattle, Washington 98115, USA

An improved understanding of the partitioning of carbon between the atmosphere, terrestrial biosphere, and ocean allows for more accurate predictions of future atmospheric CO_2 concentrations under various fossil-fuel CO_2 -emission scenarios. One of the more poorly quantified relevant processes is the interannual variability in the uptake of fossil-fuel CO_2 from the atmosphere by the terrestrial biosphere and ocean. Existing estimates, based on atmospheric measurements, indicate that

the oceanic variability is large¹⁻³. Here we estimate the interannual variability in global net air-sea CO₂ flux using changes in the observed wind speeds and the partial pressure of CO₂ (p_{CO_2}) in surface sea water and the overlying air. Changes in seawater p_{CO_2} are deduced from interannual anomalies in sea surface temperature and the regionally and seasonally varying temperature-dependence of seawater p_{CO_2} , assuming that variations in sea surface temperature reflect seawater p_{CO_2} changes caused by thermodynamics, biological processes and water mixing. The calculated interannual variability in oceanic CO₂ uptake of 0.4 Gt C yr⁻¹ (2 σ) is much less than that inferred from the analysis of atmospheric measurements¹⁻³. Our results suggest that variable sequestration of carbon by the terrestrial biosphere is the main cause of observed year-to-year variations in the rate of atmospheric CO₂ accumulation.

Interannual variations in fossil-fuel CO₂ uptake by the land biosphere and ocean have been estimated from variations in atmospheric CO₂ and ¹³CO₂ growth rates in conjunction with global carbon-cycle models¹⁻³. The combined biosphere and oceanic uptake is calculated from the difference between fossil-fuel emissions and observed atmospheric CO₂ increase. The partitioning between the land biosphere and ocean is determined from changes in atmospheric ¹³CO₂. Terrestrial CO₂ uptake by photosynthesis discriminates against the heavier carbon isotope, leaving the atmos-

phere enriched in ¹³C, while oceanic uptake shows significantly smaller fractionation, causing less change in atmospheric ¹³C/¹²C ratio. Three independent studies using this method, which is referred to as "double deconvolution calculation", suggest large interannual variability in oceanic uptake of 2.5 Gt C yr⁻¹ (2 σ ; ref. 1), 2.0 Gt C yr⁻¹ (2 σ ; ref. 2) and 1.3 Gt C yr⁻¹ (2 σ ; ref. 3) for the period from 1982 to 1995 (Fig. 1). However, no independent estimate using oceanic measurements has been made to date. Such verification is very difficult because of spatial and temporal variability in net air-sea CO₂ flux and the lack of long-term time series observations of the partial pressure of CO₂ in sea water ($p_{\text{CO}_2\text{SW}}$) on global scale.

Takahashi *et al.*⁴ have constructed a monthly mean global distribution of Δp_{CO_2} for a composite non-El Niño year using $\sim 2.5 \times 10^5$ measurements made during about 250 expeditions over the past 20 years (Δp_{CO_2} is the difference between $p_{\text{CO}_2\text{SW}}$ and the partial pressure of CO₂ in air, $p_{\text{CO}_2\text{AIR}}$). Because of the spatial and temporal sparseness of observations, the multi-year data were normalized to 1990 and interpolated onto a 4° × 5° grid per month using the surface flow field of the Princeton/GFDL general circulation model⁵. The net air-sea CO₂ flux (F) was determined from $F = kK_0\Delta p_{\text{CO}_2}$ where k is the monthly mean gas transfer velocity and K_0 is the solubility of CO₂. The gas transfer velocity was calculated from an empirical quadratic relationship with wind speed, $k = 0.39u_{\text{av}}^2 (\text{Sc}/660)^{-0.5}$ where 660 is the Schmidt number (Sc) of CO₂ in sea water at 20 °C, and u_{av} is the monthly mean climatological wind speed⁶.

Here we estimate the monthly mean CO₂ flux for each pixel for the period between 1982 and 1995 from the global climatology of Δp_{CO_2} (ref. 4), together with global records of monthly mean wind speed⁷, and sea surface temperature (SST) anomalies compared to the 1990 climatology in the following manner:

$$F_{ym} = k_{ym}K_{0,ym}\{[p_{\text{CO}_2\text{SW } 1990m} + (\partial p_{\text{CO}_2\text{SW}}/\partial \text{SST})_{1990m} \times \Delta \text{SST}_{ym - 1990m}] - p_{\text{CO}_2\text{AIR } 1990m}\}$$

where ym is the year and month in the time series between 1982 and 1995, and subscript 1990 m refers to the month in 1990. We assume that the variability in k_{ym} is related to the monthly mean wind speed variations and that the interannual changes in Δp_{CO_2} can fully be accounted for by SST variations through seasonal $p_{\text{CO}_2\text{SW}}$ -SST relationships derived for each pixel. Temporal and spatial variations in atmospheric CO₂ levels for 1990, $p_{\text{CO}_2\text{AIR } 1990m}$, are well known from extensive observations and robust interpolations⁸. The interannual variability of the net air-sea CO₂ flux for 1982-1995 calculated by this method is 0.4 Gt C yr⁻¹ (2 σ) (case 1 in Fig. 4).

For each 4° × 5° pixel, the seasonal $p_{\text{CO}_2\text{SW}}$ -SST relationship is determined from least-squares linear fits of monthly climatological $p_{\text{CO}_2\text{SW}}$ and SST values⁴ for three periods, January-April, May-August, and September-December. The $p_{\text{CO}_2\text{SW}}$ -SST relationship has a thermodynamic component and an empirical part incorporating $p_{\text{CO}_2\text{SW}}$ changes due to the influence of lateral and vertical mixing of water with different levels of total inorganic carbon (ΣCO_2) (the transport effect), photosynthesis and respiration in the surface layer (the biological effect), and air-sea gas exchange. The thermodynamic component is caused by changes in solubility of CO₂ with temperature. It can be well approximated by a change of 4.23% in $p_{\text{CO}_2\text{SW}}$ per °C for the surface ocean⁹. The biological and transport effects are caused by changes in ΣCO_2 and, to a lesser degree, in alkalinity, which in turn affect $p_{\text{CO}_2\text{SW}}$. Air-sea gas transfer will generally have a smaller effect on $p_{\text{CO}_2\text{SW}}$ because of the carbonate-buffer factor.

Either directly or indirectly, the thermodynamic, transport and biological effects are correlated with temperature. The effects are of similar magnitude but often act in opposite directions, and differ geographically and seasonally. Several general trends in the $p_{\text{CO}_2\text{SW}}$ -SST relationship are found in the world oceans (Fig. 2)⁹⁻¹⁸. At high latitudes, ΣCO_2 at the surface increases during seasonal cooling due

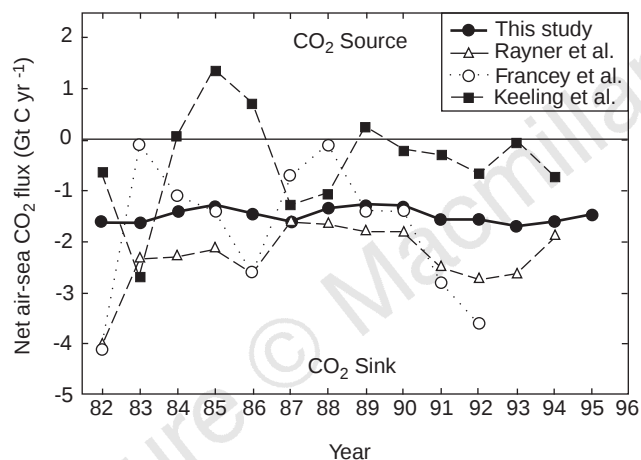


Figure 1 Comparison of net air-sea CO₂ fluxes, 1982-95. Shown are the values from this study (case 1 in Fig. 4) compared with those of Francey *et al.*¹, Keeling *et al.*² and Rayner *et al.*³. Negative values correspond to net oceanic CO₂ uptake. Net oceanic uptake deduced from atmospheric deconvolution calculations¹² is uncertain by about ± 0.5 to 1 Gt C yr⁻¹. The double deconvolution calculations give interannual variability in fossil-fuel carbon uptake while our analyses shows interannual variability in air-sea fluxes. Therefore, 0.6 Gt C yr⁻¹ was added to the double deconvolution for an absolute comparison between the methods to account for a steady-state natural efflux caused by the weathering cycle²⁵. Our estimated net air-sea CO₂ fluxes are subject to errors due to uncertainties in the following independent sources³: (1) The gas transfer velocity; the estimated fluxes are subject to an error of up to 100% depending on the choice of relationships between gas exchange and wind speed. (2) The dependence of k on the wind speed variability; the relationship between gas exchange and wind speed used⁶ takes this into account but our calculated net fluxes could be in error by $\sim 10\%$ due to uncertainty in this effect. (3) Errors of $\pm 5 \mu\text{atm}$ in Δp_{CO_2} due to the normalization of 20 years of data to a reference year 1990⁴; this is equivalent to an error of up to 75% of the estimated fluxes regardless of the choice of k . (4) The skin temperature effect on Δp_{CO_2} ; its effect on the global Δp_{CO_2} is not taken into account in this study due to limited verification of this effect. (5) The empirical $\Delta p_{\text{CO}_2\text{SW}}$ -SST relationships; 20% (2 σ) uncertainties in derived seasonal $\Delta p_{\text{CO}_2\text{SW}}$ -SST relationships result in uncertainties of ± 0.2 to 0.3 Gt C yr⁻¹ in the estimated net fluxes. (6) Applying seasonal $\Delta p_{\text{CO}_2\text{SW}}$ -SST relationships to deduce interannual changes. However, except for (5) and (6), none of these uncertainties are thought to significantly influence the relative magnitude of interannual variations.

to the convective mixing of deep waters rich in ΣCO_2 (Fig. 2a–d). In this case, the thermodynamic and transport effects are out of phase, and $p_{\text{CO}_2\text{SW}}$ reductions by seasonal cooling are opposed and frequently overtaken by increases due to vertical mixing. Phytoplankton blooms at high latitudes can cause rapid $p_{\text{CO}_2\text{SW}}$ decreases in springtime. The initiation of blooms corresponds with SST increase resulting from a combination of increased springtime irradiance and stratification. Therefore, the observed $p_{\text{CO}_2\text{SW}}$ –SST relationships for high-latitude waters reported in the literature vary seasonally and geographically over a wide range from -6% per $^{\circ}\text{C}$ in winter to $+4\%$ per $^{\circ}\text{C}$ in summer^{9,13}. In temperate and tropical oceans^{15–17} where the mixed layer is shallow and vertical mixing is weak, the $p_{\text{CO}_2\text{SW}}$ –SST relationship is seasonally less variable and is generally positive ranging from $+1$ to $+4\%$ per $^{\circ}\text{C}$ (Fig. 2e–f). In the eastern equatorial Pacific Ocean (EPO) the distribution of $p_{\text{CO}_2\text{SW}}$ is to a large extent controlled by the upwelling intensity of subsurface water with high ΣCO_2 and changes in $p_{\text{CO}_2\text{SW}}$ are thus strongly inversely correlated with changes in SST^{16,18}. The $p_{\text{CO}_2\text{SW}}$ climatology⁴ is for a non-El Niño year so we use a value of -6% per $^{\circ}\text{C}$ that was obtained from measurements made during both the warm and cold phase of the El Niño/Southern Oscillation (ENSO) for the region from 10°N to 10°S and 80°W to 150°W ¹⁸. In general, because of transport and biology, the $p_{\text{CO}_2\text{SW}}$ –SST relation-

ships used for each pixel in this study are different and usually less than the thermodynamic effect of 4.23% per $^{\circ}\text{C}$.

The important assumption in our approach—that the $p_{\text{CO}_2\text{SW}}$ –SST relationships derived from the annual $p_{\text{CO}_2\text{SW}}$ climatology⁴ can be used to infer interannual variations in fluxes—can be checked against observations from the eastern EPO, and the Bermuda Atlantic Time-series Study (BATS) and the Hawaiian Ocean Time-series (HOT) sites. The time-series stations have monthly records of SST and $p_{\text{CO}_2\text{SW}}$ spanning four to six years that were not incorporated in the $p_{\text{CO}_2\text{SW}}$ climatology⁴, while the eastern EPO fluxes are estimated from bi-annual observations¹⁸. There is broad agreement between the modelled and observed $p_{\text{CO}_2\text{SW}}$ –SST relationships (Fig. 3c and e), and between our modelled net fluxes and those observed in the eastern EPO¹⁸ (Fig. 3a), BATS¹⁷, (Fig. 3b) and HOT¹⁹ (Fig. 3d) but our model does appear to underestimate the interannual variability to some extent.

The derived seasonal $p_{\text{CO}_2\text{SW}}$ –SST relationships have an average of 20% (2σ) uncertainty; sensitivity studies were performed by adjusting the seasonal relationships by 20% in such a way to estimate possible annual maximum and minimum air–sea CO_2 flux. For a maximum net flux for an individual year (the lower boundary of the shaded area in Fig. 4a) seasonal $p_{\text{CO}_2\text{SW}}$ –SST relationships for all the pixels where signs of the $p_{\text{CO}_2\text{SW}}$ –SST relationships and SST

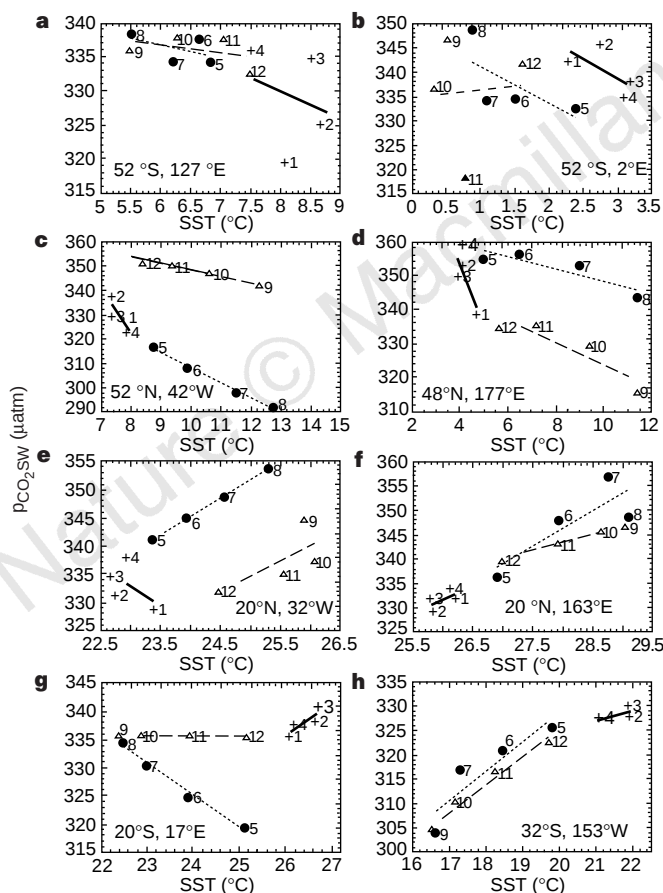


Figure 2 Examples of $\Delta p_{\text{CO}_2\text{SW}}$ –SST relationships for pixels ($4^{\circ} \times 5^{\circ}$) in our analysis. **a, b**, Southern Ocean; **c, d**, high-latitude northern oceans; **e–h**, subtropical gyres. The numbers by the symbols refer to the month (1 = Jan.). The January–April points are shown as plus symbols with a solid line showing the linear regression line used in the $\Delta p_{\text{CO}_2\text{SW}}$ –SST relationship; the May–August data are solid circles and the dotted line, and September–December data are the open triangles and dashed lines. The average regional relationships for January–April, May–August, and September–December are, respectively (in % per $^{\circ}\text{C}$): -0.7 , -2 , -1.5 for the Southern Ocean; -2.2 , -1.2 , -1.7 for the high northern latitudes ($>40^{\circ}\text{N}$); and 0.7 , 1.8 , and 1% for the subtropical zone (40°S – 40°N).

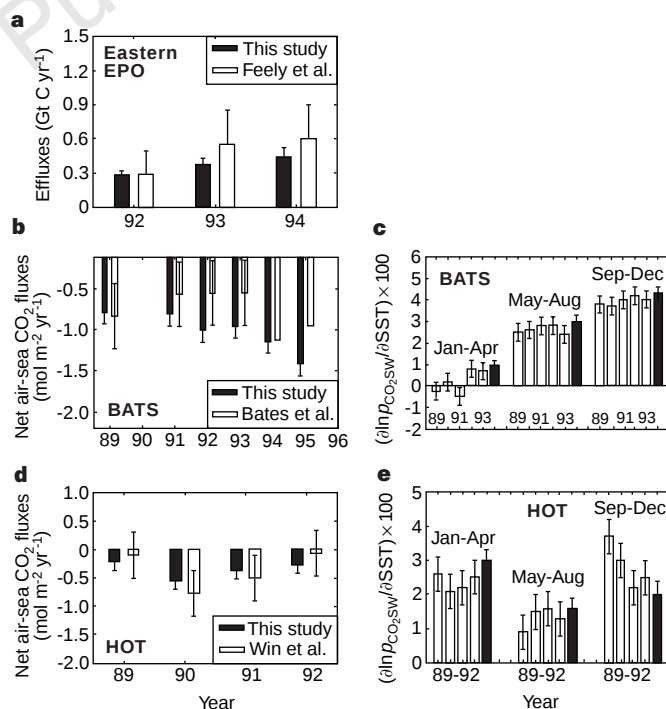


Figure 3 Comparisons of modelled net air–sea CO_2 fluxes (filled bars) with multi-year observations (open bars). **a**, In the eastern equatorial Pacific Ocean (EPO) (10°N – 10°S , 80°W – 135°E); **b**, at BATS ($31^{\circ}50'\text{N}$, $64^{\circ}10'\text{W}$); and **d**, at HOT (22°N , 158°W). Net reported equatorial effluxes¹⁸ were scaled down to 0.3 Gt C in 1992, 0.55 Gt C in 1993, 0.6 Gt C in 1994, because of small differences between NCEP/NCAR and EWMF winds used in ref. 18. At the BATS site, net air–sea CO_2 fluxes for the period 1989–93 were estimated using calculated $p_{\text{CO}_2\text{SW}}$ from total alkalinity (TA) and total inorganic carbon (ΣCO_2) using thermodynamic relationships, whereas net CO_2 fluxes for 1994–95 were estimated using direct measurements of $p_{\text{CO}_2\text{SW}}$. At the HOT site, net air–sea CO_2 fluxes for the period 1989–92 were estimated from calculated $p_{\text{CO}_2\text{SW}}$ from TA and ΣCO_2 . Error bars for these studies indicate uncertainties in net fluxes due to measurement errors in TA and ΣCO_2 (± 4 and $\pm 2\text{ }\mu\text{mol kg}^{-1}$, respectively). Error bars for our study were estimated from uncertainties of the fits between climatological $p_{\text{CO}_2\text{SW}}$ and SST in the pixel including the time series station. **c** and **e**, The measured (open bar) and modelled (filled bar) $p_{\text{CO}_2\text{SW}}$ –SST relationships ($[\Delta \ln p_{\text{CO}_2\text{SW}} / \Delta \text{SST}] \times 100 = \%$ per $^{\circ}\text{C}$) at BATS and HOT for different years.

anomalies ($\Delta \text{SST}_{\text{ym}-1990\text{m}}$) are the same were decreased by 20% ($= 0.8 \times \partial p_{\text{CO}_2\text{SW}} / \partial \text{SST}$), while $p_{\text{CO}_2\text{SW}}$ -SST relationships for all the pixels where signs of the relationships and SST anomalies are the opposite were increased by 20%. For a minimum net flux (the upper boundary of the shaded area in Fig. 4a), the opposite condition to the maximum flux was used. The maximum magnitude of interannual net air-sea CO_2 flux variability is estimated by assuming the maximum air-sea fluxes are followed by minimum annual air-sea fluxes. This yields an interannual variability of 0.6 to 0.8 Gt C yr^{-1} .

The derived $p_{\text{CO}_2\text{SW}}$ -SST relationships were also compared with reported regional relationships⁹⁻¹⁸. The relationships obtained with the two methods are in good agreement, when seasonal relationships for each pixel are averaged over the same areas. The average and interannual variability of net air-sea CO_2 flux for 1982-95 obtained for this method (case 2) is similar as well (Fig. 4).

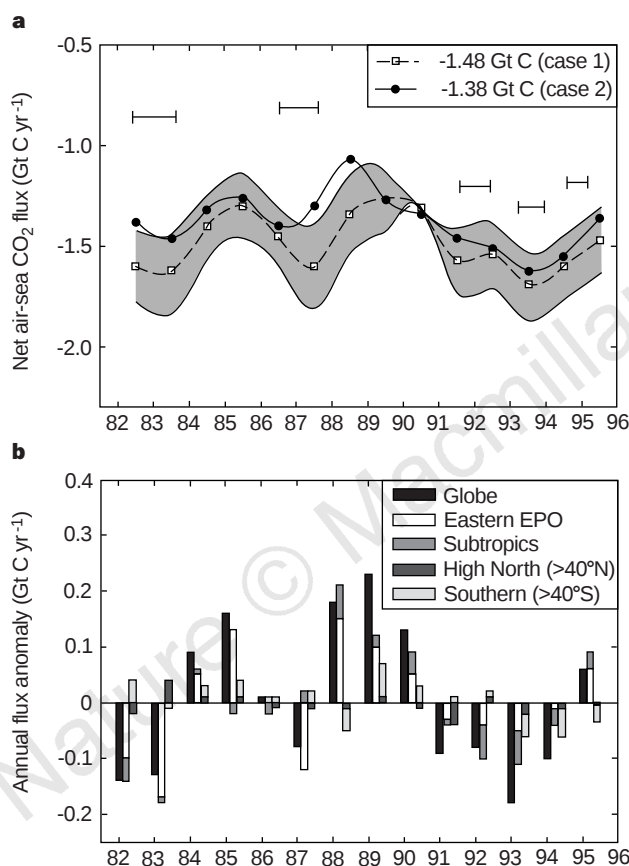


Figure 4 Net global air-sea CO_2 flux and annual flux anomaly. **a**, Net global air-sea CO_2 fluxes from 1982-95 for two different scenarios discussed in the text. For case 1, derived seasonal relationships were used. The shaded area depicts maximum and minimum air-sea CO_2 flux caused by 20% uncertainty in derived seasonal relationships. Case 2 uses $p_{\text{CO}_2\text{SW}}$ -SST relationships reported in the literature. The following reported yearly mean temperature coefficients are used: -4% per $^{\circ}\text{C}$ (-4) in the North Atlantic and Pacific (40°N - 70°N)^{9,10}; 2.5% per $^{\circ}\text{C}$ (1 to 4) in the subtropical gyres (40°S - 40°N except the eastern EPO)¹²⁻¹⁷; -6% per $^{\circ}\text{C}$ (-4 to -7) in the eastern EPO (10°N - 10°S , 80°W - 150°W)^{16,18}; -1.5% per $^{\circ}\text{C}$ (-1.5) in the Southern Ocean^{9,11,13,14}. Values (% per $^{\circ}\text{C}$) in parentheses are the ranges in reported literature values. Horizontal bars indicate times of El Niño events. The 14-year mean net global fluxes for cases 1 and 2 are listed in the legend. The curves are spline fits. **b**, Annual flux anomalies for the globe, the eastern EPO (10°N - 10°S , 80°W - 150°W), subtropical, and high-latitude oceans calculated from the results of case 1. The flux anomalies are differences from a 14-year average of $-1.48 \text{ Gt C yr}^{-1}$ for the globe, or 0.3 Gt C yr^{-1} for the eastern EPO, of $-0.3 \text{ Gt C yr}^{-1}$ for subtropical gyres except the eastern EPO, of $-0.7 \text{ Gt C yr}^{-1}$ for northern high-latitude regions ($>40^{\circ}\text{N}$), and of $-1.0 \text{ Gt C yr}^{-1}$ for the Southern Ocean ($>40^{\circ}\text{S}$). Negative anomalies correspond to greater net air-sea CO_2 uptake.

Annual flux anomalies differ regionally, and much of the global anomaly can be attributed to changes in the eastern EPO (Fig. 4b). The trend appears to change in the early 1990s, with smaller anomalies in the eastern EPO and greater changes in the subtropics. In this analysis interannual variability of net CO_2 flux in the Southern Ocean is significantly smaller than variability in the eastern EPO. As the SST climatology²⁰ is based almost solely on satellite observations with little *in situ* verification in the Southern Ocean, it remains a possibility that the small flux anomalies are caused by biases in interannual SST anomaly in this region.

The interannual variations in wind speeds account for $<10\%$ of the total variations in net air-sea CO_2 flux except for 1982-83 and 1986-87 when strong El Niño events took place. During these events, wind speed anomalies that are predominantly in the equatorial region account for 20-30% of the annual net flux anomalies.

The sharp contrast between our modelled net air-sea CO_2 flux variations and those determined from atmospheric double deconvolution calculations could be caused by several factors. Interannual changes in growth of C_3 and C_4 plants that have different fractionation factors for photosynthesis ($\delta^{13}\text{C} = -10\%$ to -15% for C_4 and -25% to -30% for C_3) can cause errors of about $\pm 1 \text{ Gt C yr}^{-1}$ in estimating terrestrial and oceanic carbon uptakes based on atmospheric observations^{21,22}. Inaccurate knowledge of the isotopic ratios of reservoirs, and fractionation factors during exchange between reservoirs, contributes to the uncertainty¹⁻³, as does the lack of sufficient atmospheric time series of ^{13}C and errors in atmospheric-transport models. Figure 1 shows three different double deconvolution analyses with large differences in interannual variability. The more recent work³ shows significantly smaller interannual variability, but still larger than we estimate (Fig. 1).

Our method may underestimate the amplitude of interannual variability in net air-sea flux. The Δp_{CO_2} climatology⁴ smooths the regional variability because of normalization of 20 years of data to the virtual year 1990. The interpolation schemes of $p_{\text{CO}_2\text{SW}}$ and SST data in space and time may cause a mismatch between $p_{\text{CO}_2\text{SW}}$ and SST. The simple $p_{\text{CO}_2\text{SW}}$ -SST relationships might not capture all $p_{\text{CO}_2\text{SW}}$ variability such as that caused by interannual changes in the freshwater fluxes, particularly in the EPO. During ENSO events, low-salinity surface water with low Δp_{CO_2} moves from the western EPO into the central and eastern EPO²³. The $p_{\text{CO}_2\text{SW}}$ climatology⁴ does not include coastal regions, and interannual variations in ENSO-induced coastal upwelling and associated $p_{\text{CO}_2\text{SW}}$ variations along the west coast of America may not be properly accounted for. Temperature anomalies might not capture the full biological effect, such as functional changes in ecosystems.

Atmospheric deconvolution models¹⁻³ suggest that ENSO events have a large influence on the interannual variability in net air-sea CO_2 flux. Our results show the same, but the magnitude of the interannual variability is much smaller. The use of $p_{\text{CO}_2\text{SW}}$ -SST relationships is a first step in estimating interannual variations in net global air-sea CO_2 flux. Recent work using a three-dimensional global ocean circulation model by Le Quéré *et al.*²⁴ also suggests that the interannual variability in net air-sea CO_2 flux is smaller than inferred from atmospheric deconvolutions, and that most of the variability is caused by changes in the eastern EPO. In the future, the use of satellite-derived observations of ocean colour, wind and SST, along with improved algorithms to relate $p_{\text{CO}_2\text{SW}}$ to these variables, should improve temporal and spatial extrapolations of $p_{\text{CO}_2\text{SW}}$ fields and thus increase our confidence in estimates of interannual variability in net air-sea CO_2 flux. □

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Correspondence and requests for materials should be addressed to K.L. (e-mail: lee@aoml.noaa.gov).

Seismic and hydrothermal evidence for a cracking event on the East Pacific Rise crest at 9° 50' N

Robert A Sohn*, Daniel J. Fornari†, Karen L. Von Damm‡, John A. Hildebrand* & Spahr C. Webb*

* Scripps Institution of Oceanography, 8602 La Jolla Shores Drive, La Jolla, California 92093-0205, USA

† Geology & Geophysics Department, Woods Hole Oceanographic Institution, Woods Hole, Massachusetts 02543, USA

‡ Department of Earth Sciences and Institute for the Study of Earth, Oceans, and Space, University of New Hampshire, Durham, New Hampshire 03824-3589, USA

Interaction between the hydrothermal system and the axial magma chamber at a mid-ocean ridge spreading centre takes place in a boundary layer of crust that separates circulating sea water from basaltic melt¹. The nature of heat flow through this region is critical because it determines the pressure–temperature conditions of the water–rock interaction and regulates the total heat flux through the system². Here we combine seismic, thermal and chemical time-series data from high-temperature vents on the

East Pacific Rise axis at 9° 50.2' N to link a microearthquake swarm with changes measured in vent fluids. Four days after the earthquake swarm opened fractures near the base of the circulation system, a sudden increase in fluid temperature in the overlying 'Bio9' black-smoker vent was observed. Temperatures peaked at the vent 11 days after the swarm and gradually declined back to just above pre-swarm levels (365 °C) over the next 70 days. These observations are consistent with the Bio9 hydrothermal

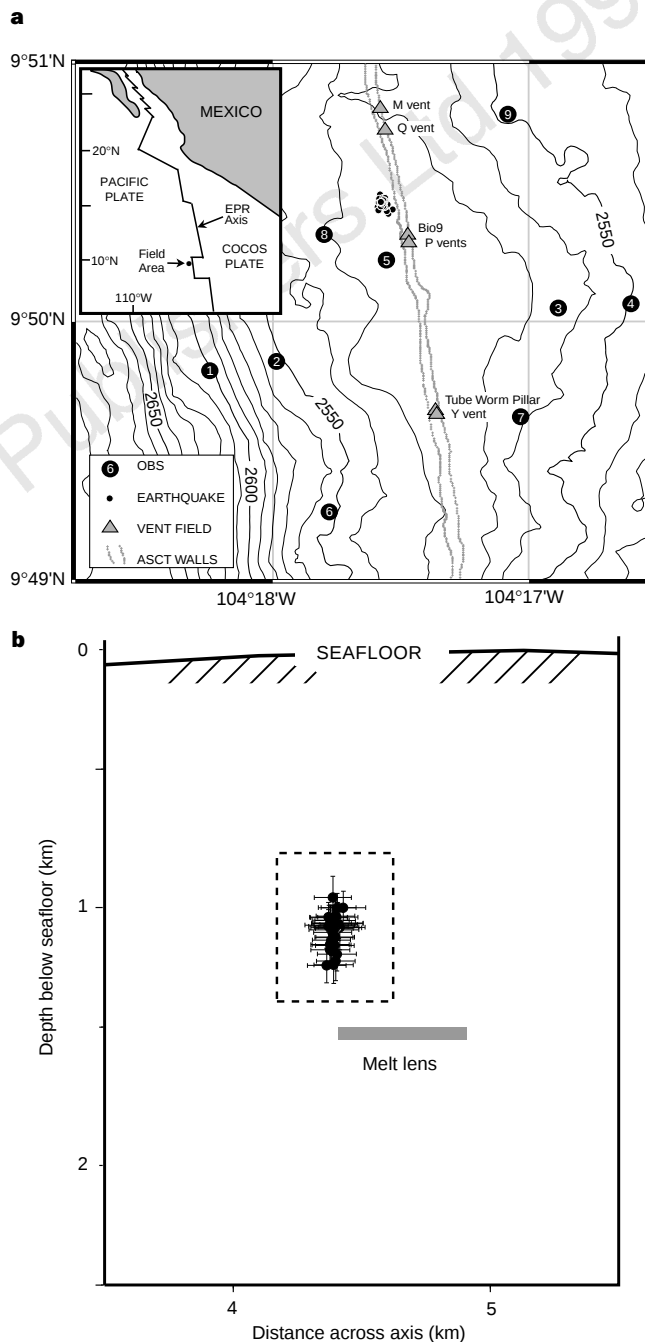


Figure 1 Study area maps. **a**, Location map of the East Pacific Rise crest near 9° 50' N showing vent locations, seismometer locations, and relocated microearthquake epicenters. Bathymetric contours are based on multibeam data and are in metres. Walls of the axial summit collapse trough (ASCT) are shown. Inset map showing location of the study area, and ridge and transform plate boundary geometry. **b**, Cross-sectional view of relocated earthquakes. Error bars represent relative 1σ limits as determined by the bootstrap method of Shearer⁷. Absolute 1σ limit of hypocentres denoted by the dashed box. Depth and width of melt lens are taken from Kent *et al.*⁸

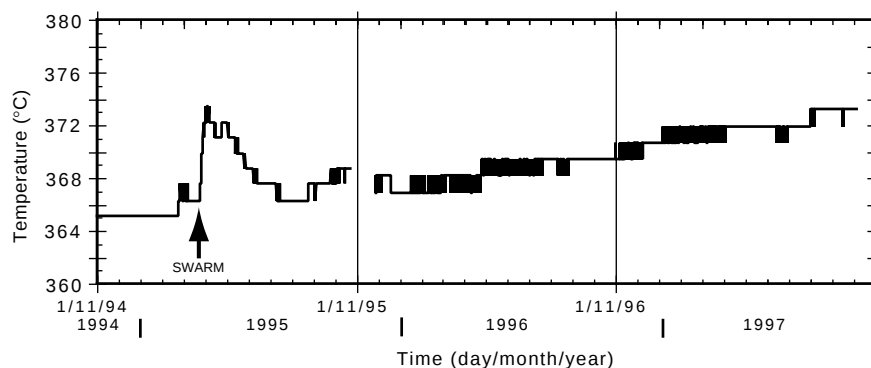


Figure 2 Time-series temperature data for fluids from Bio9 vent collected between 1994 and 1997, from Fornari *et al.*⁹ Fluid temperatures were stable for ~4 months before the 22 March, 1995 seismic swarm (denoted by arrow). Tempera-

ture measurements during 1993–95 at P vent were not successful because the probes were either destroyed by hot vent fluids or buried by toppled-over chimneys.

system tapping a previously isolated region of crust, and an upflow fluid residence time of 4 days, compared to previous lower-resolution estimates of 3 years or less³.

In March 1995 we deployed a small aperture array of nine ocean-bottom seismometers (OBSs) on the axis of the East Pacific Rise (EPR) (Fig. 1a). This site experienced volcanic eruptions in 1991⁴ and 1992⁵. The spacing and locations of the OBSs were chosen to provide good resolution for microseismic events in the upper 2 km of crust. On 22 March an intense swarm of seismic activity was recorded by the array, with 162 identifiable events occurring in less than 3 hours, followed by a smaller swarm of 25 events, 22 days later. Quasi-continuous, low-level seismic activity was observed throughout the deployment beneath the Tube Worm Pillar and Y vents⁶ (Fig. 1a), but no other swarms were seen in 105 days of recording. Hypocentres were determined for 92 of these small events (local magnitudes between -1 and 0) with a three-dimensional (3D) grid-search method, forming a 'cloud' of seismicity beneath the ridge axis at 1–2 km depth. Many of the swarm events generated remarkably similar waveforms in the OBS records, suggesting that they shared a common source mechanism and location. To characterize better the swarm seismicity, we applied the cross-correlation/relocation method of Shearer⁷ (modified to allow for a 3D velocity model), which exploits waveform similarities to obtain high-precision estimates of relative event locations. Fifty-five of the 92 located events were similar enough to allow the application of this method. The relocated hypocentres lie within a tall, thin volume of crust with a diameter less than or equal to the relative location error threshold of 100 m, and a height of ~300 m (Fig. 1b). The deepest event (1.1 km) is located ~300 m above the top of the axial magma chamber (AMC) inferred from multichannel seismic data collected in 1987⁸. Within the standard error, the relocated hypocentres (Fig. 1b) are coincident in *x-y* position, but a vertical spread of at least 200 m is required to fit the relative travel times. The seismic station coverage and first-arrival signal-to-noise ratio were insufficient to allow the determination of focal plane solutions from individual events, but a composite solution based on stacked waveforms suggests that the earthquakes were generated by oblique normal faulting.

Bio9 vent fluid temperatures, recorded almost continuously since December 1993 with an accuracy of $\pm 1^\circ\text{C}$, were stable at $\sim 365^\circ\text{C}$ for approximately a year before the seismic event⁹. On 26 March, 1995, four days after the swarm, vent fluid temperatures at Bio9 began to increase at a rate of 1°C per day for 7 days. After peaking at 373°C , fluid temperatures gradually decayed back to just above pre-swarm levels over the next 70 days (Fig. 2). After this event, Bio9 vent fluids entered a prolonged period of steady increase, rising back to 373°C by the end of 1997⁹. The time delay between the onset of the seismic swarm and the abrupt increase of Bio9 vent fluid temperatures provides evidence that the upflow limb of the circulation system feeding this vent in 1995 had a residence time of ~4

days. This provides much finer resolution than previous estimates of fluid residence times in mid-ocean ridge hydrothermal fluid convective systems based on radionuclide data³, which suggested the time interval between fluid reaction with the substrate and sea-floor venting was less than 3 years.

Changes in Bio9 fluid chemistry during 1995 are consistent with a deepening of the water-rock reaction zone, although the long interval between chemical samples makes direct correlation with the seismic event difficult. The last fluid samples collected from high-temperature vents at $9^\circ 50' \text{N}$ before the seismic swarm were taken in October 1994. At this time, neither Bio9 nor P vent (located ~40 m south; Fig. 1) had made the transition to venting fluids with chloride concentrations greater than sea water. As fluids from both vents have chloride concentrations that are distinct from sea water, they indicate that phase separation has occurred and may be further used to constrain the pressure-temperature conditions of the reaction zone. In 1994, P vent had significantly greater chloride concentrations than Bio9 (Fig. 3), suggesting it was being fed by a separate, deeper reaction zone despite the close proximity of the two vents on the sea floor. Silica contents from the two vents are consistent with this interpretation (Fig. 3). When next sampled in November 1995, seven months after the swarm, P-vent fluid chloride contents had increased by 17% (crossing the seawater value and becoming a brine), while its silica content remained fairly stable. During this same period Bio9 chloride concentrations increased by 52% (still less than sea water), and the silica content increased, suggesting that the reaction zone supplying Bio9 fluids had deepened.

Thus we find that the seismic swarm had the effect of triggering a short-term 7°C temperature increase in the overlying Bio9 vent fluids, and that the fluid chloride and silica contents increased markedly. The thermal anomaly generated by the microearthquakes indicates that they established a fracture network that allowed circulating fluids to penetrate fresh rock, or perhaps opened communication with previously isolated fluids. Although a number of source mechanisms can be put forward, the vertical fracture plane that we imaged above the west margin of the melt lens (Fig. 1b) suggests that the earthquakes were triggered by subsidence of the local magma chamber lid. Contraction or deflation of the AMC would increase normal stresses across vertical faults in our hypocentral regions, and could trigger a swarm such as we observed when the stresses reach the rock strength limit.

We interpret the evolution of the magmatic and hydrothermal system beneath the EPR crest near $9^\circ 50' \text{N}$ over the past six years as follows. (1) Dykes were intruded beneath the EPR axis in 1991⁴ and 1992⁵, and then were rapidly cooled by hydrothermal fluids, a trend that is borne out by the chemical time-series data¹⁰ and thermal models of dyking¹¹. The short-term chemical and heat fluxes generated by the 1991/1992 events dissipated well before the fracturing event in March 1995. (2) Several years after the volcanic

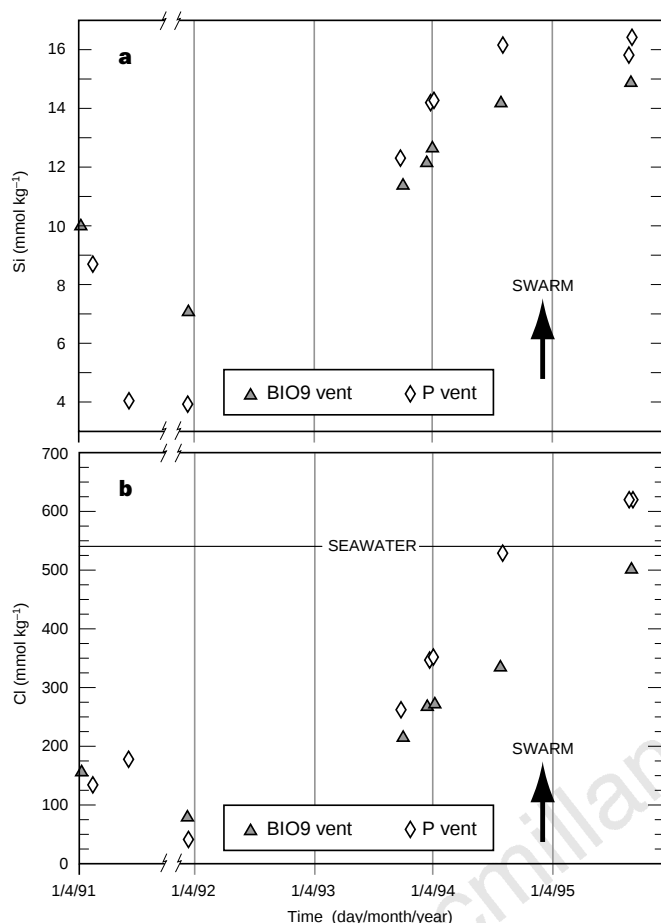


Figure 3 Contents of vent fluids. The silica (a) and chloride (b) contents of vent fluids sampled from the Bio9 (triangles) and P (diamonds) vents. Local bottom sea water contains 540 mmol kg^{-1} Cl and $0.16 \text{ mmol kg}^{-1}$ Si. Although the Si and Cl contents for both vents have been gradually increasing since 1992, there are large changes, especially in Cl for Bio9 fluids during the time interval in which the seismic swarm occurred, suggesting a marked change in the water-rock reaction conditions. The unusual compositions early in the time series are interpreted to result from shallow, dyke-driven circulation which had ceased by December 1993.

activity, the silica and chloride contents of Bio9 vent fluids remained low compared to P vent, despite their close spatial proximity on the sea floor. The chemical divergence of Bio9 and P-vent fluids beginning in December 1993 suggests that the plumbing system for these vents became hydrologically isolated at that time, and that the circulation system feeding P vent deepened relative to that feeding the Bio9 vent¹⁰. (3) On 22 March 1995 stresses above the west margin of the AMC became large enough to trigger fracturing and hydrothermal penetration of a 200–300-m-tall column of rock, generating a temperature anomaly that was observed at the sea floor 4 days later. The temperature anomaly represents heat extracted from previously untapped rock and perhaps from fluid reservoirs. Vent-fluid chemical changes during this period are consistent with a deepening of the hydrothermal reaction zone. (4) Vigorous hydrothermal activity persists at $9^{\circ} 50' \text{ N}$ to this day, more than seven years after the last volcanic event. Our results demonstrate that this activity is fuelled by a dynamic convection system that responds quickly to seismic perturbations, and with reaction-zone conditions that continually evolve as heat is removed from the shallow crust. □

Methods

The relative locations of earthquakes in the seismic swarm were determined by applying a modified version of the method developed by Shearer⁷. Waveform

cross-correlation was used both to characterize the degree of waveform similarity and to provide differential times between pairs of seismograms for correlated events. Cross-correlation was performed separately for both compressional and shear arrivals, using windows of 0.55 s and 0.8 s, respectively, after first applying a 12–35-Hz bandpass filter to the records. Event pairs with average correlation coefficients >0.7 were considered to be doublets. Differential compressional and shear arrival times at each instrument were calculated by finding the peak of the cross-correlation signature for each doublet. A grid of relative shear and compressional arrival times was constructed for each instrument with horizontal spacings of 70 m and a vertical spacing of 53 m about the centre of the swarm as determined by 3D grid-search locations, with total dimensions of $1.96 \times 1.96 \times 1.48 \text{ km}$ in the x -, y - and z -dimensions, respectively. Differential travel times within the grid were estimated using the compressional velocity model of Vera *et al.*¹² and a shear model determined from compliance measurements (W. Crawford, personal communication). The L1 norm best-fit differential location of each doublet was found with a grid-search method, with sub-grid space precision achieved by linear interpolation of the travel times between grid points. Finally, the set of differential locations between individual event pairs was inverted to obtain a final set of relative locations. Events with relative 1σ errors $>100 \text{ m}$ were discarded.

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Correspondence and requests for materials should be addressed to R.A.S. (e-mail: rsohn@ucsd.edu).

Navigation through vector addition

A. S. Etienne, R. Maurer, J. Berlie, B. Reverdin, T. Rowe, J. Georgakopoulos & V. Séguinot

Laboratoire d'Éthologie, FPSE, Université de Genève, 54 route des Acacias, 1227 Carouge, Switzerland

During short foraging excursions away from their home, central place foragers update their position relative to their point of departure by processing signals generated by locomotion. They therefore can home along a self-generated vector without using learned references. In rodents^{1–5} and other mammals^{6,7}, this path integration process (dead reckoning) can occur on the basis of purely internal signals, such as vestibular⁸ or proprioceptive (re)afferences⁶. We report here that hamsters are also capable of

proceeding to a previously learned feeding site through vector information from locomotion only. The subjects compute⁹ the direction and distance to the goal by subtracting their current-position vector from the stored nest-to-goal vector. This computation pertains to locations *per se* and therefore occurs in absolute space, independently of landmark objects. If available, prominent visual cues merely serve to confirm the path planned through the addition of self-generated vectors, whereas visual as well as non-visual references confirm that the subject has arrived at the goal site.

To investigate whether self-generated vector information plays a more general role¹⁰ in navigation than allowing central place foragers to return to their home bases, we tested the capacity of golden hamsters (*Mesocricetus auratus* W.) to reach a familiar feeding place through variable paths, in the absence of unambiguous direction and position cues. In a first experiment ('Light'), we trained six subjects to proceed along two different routes from their peripheral nest to a baited cylinder at a constant location in a large, optically shielded arena (Fig. 1). The baited cylinder formed, together with three identical, but unbaited, cylinders, a symmetrical landmark array around the arena centre. In six different test trials, we led the animals in darkness along six new routes from the nest exit to four different points at the arena periphery. From there they had to reach the goal cylinder autonomously in the fully lit symmetrical test space. Under these conditions, the subjects could identify the goal cylinder only through its position, which they might derive from their own current position, known through path integration.

Our six subjects proceeded to the landmark array and climbed into a cylinder in 97% of the test trials. Table 1 (upper part) shows that in 89% of these trials, the animals chose first the cylinder at the correct goal location. After having filled their cheek pouches, the animals returned fairly directly to the (invisible) nest entrance. For particular subjects, the success rate varied from 67 to 100%. The mean performance remained high for all test trials except for trial 5, in which the outward journey was the longest¹¹ if we consider both the guided trip along the periphery and the autonomous path across the centre of the arena. The success rate rose again to 100% in trial 6, where the (longest) guided outward journey led to the same end-point as one of the two training trials.

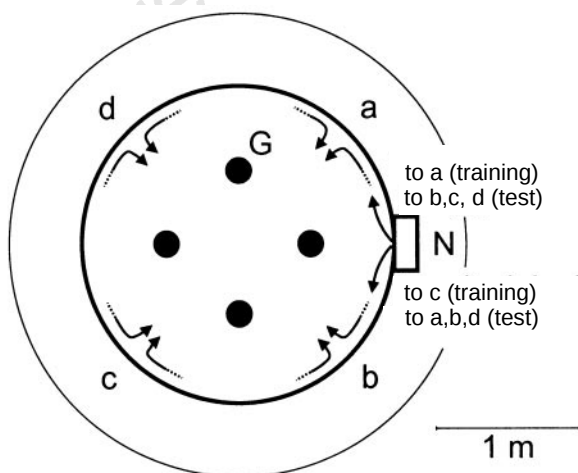


Figure 1 Apparatus. The arena (inner circle: diameter, 220 cm; height of black wall, 45 cm) is surrounded by a white cardboard enclosure (outer circle) and covered by an artificial white cloth ceiling. Four identical grey cylinders (black dots: diameter, 16 cm; height, 28 cm) are placed symmetrically around the arena centre, at a distance of 50 cm. Apart from the control trials, the cylinder at the goal location G is baited. A nest box (N), where the animal establishes its granary, is fixed to the arena wall. The arrows represent the beginning (at the nest exit) and the end (at the peripheral points a, b, c or d) of the guided outward journeys along the arena wall in training and test trials.

Figure 2 shows that in trials 1, 3 and 5, in which the animals had to walk through the landmark array, they passed under a nearby cylinder (and often paused there) in more than half (57%) of the trials, or even performed considerable detour loops before arriving at the goal. In trials 2, 4 and 6, where the animals did not have to cross the arena to reach the correct cylinder, they proceeded fairly directly from the arena periphery to the goal.

Three control tests with an unbaited cylinder at the goal location were carried out on five new subjects to exclude the role of uncontrolled external references. (1) Control for room cues. Before the beginning of a trial, we rotated the arena and nest by 90°. Unrotated room cues should therefore lead the animal to the usual goal location with respect to room space, whereas arena cues and/or path integration should lead to the usual goal location with respect to the internal arena space and the current nest location. In tests with an anticlockwise arena rotation, all five subjects significantly chose the cylinder specified by path integration and/or arena cues. (two animals chose this cylinder in 5 trials out of 6, and three animals chose it in 4 trials; $P < 0.01$ and $P < 0.05$ respectively, binomial test). After a clockwise arena rotation, four animals significantly chose the above-mentioned cylinder. (One animal chose this cylinder in 5 trials, three animals in 4 trials, and one animal in 3 trials; $P < 0.01$, $P < 0.05$ and $P > 0.05$ respectively.) In all, the animals located the goal site through path integration and/or arena cues in 70% of a total of 60 trials. (2) Control for both room cues and arena cues. Before the beginning of the trial, we rotated the arena anticlockwise and the nest clockwise by 90° with respect to their standard position. Under these conditions, room cues, arena cues and path integration should lead the subject to three different cylinders. Four subjects out of five significantly chose the cylinder specified by path integration. (One animal chose this cylinder in 6 trials out of six, three animals in 4 trials, and one animal in 3 trials; $P < 0.001$, $P < 0.05$, $P > 0.05$ respectively.) In all, this cylinder was chosen in 70% of 30 trials. (3) Control with an extended outward journey. Four of the five control subjects underwent a third control experiment, testing whether an extended guided outward trip would disrupt the animals' capacity to select the goal cylinder through path integration¹¹. In each of the six trials, we extended the guided outward journey by leading the animal three times

Table 1 Performance in experiment Light and Dark

Trial no.	1	2	3	4	5	6	Total
	Light						
Performed	5	6	6	6	6	6	35
Successful	5	6	6	5	3	6	31
% success	100	100	100	83	50	100	89
	Dark						
Performed	4	5	3	5	3	4	24
Successful	4	5	3	5	3	3	23
% success	100	100	100	100	100	75	96

The numbers at the top represent the six test trials that each of the six subjects passed in succession; the curved arrow inside each circle represents the guided path from the nest (rectangle) along the arena periphery; the dot indicates the goal cylinder. The results are presented in terms of the number of trials in which the subjects proceeded to the landmark array and climbed into a cylinder (Performed), the number of trials in which the subjects' first choice was the cylinder at the goal location (Successful), and the percentage of successful trials relative to the number of performed trials (% Success).

around the arena periphery. In these conditions, we did not expect the animal to choose a particular cylinder unless it depended on arena or room cues. In all, our four subjects chose the cylinder specified by arena cues in 5 trials out of 24, the cylinder specified by room cues in 6 trials, and the cylinder specified by (unjammed) path integration in 5 trials. These results are not different from a random distribution.

Taken together, the three control experiments confirm that path integration, rather than room or arena cues, allows our subjects to identify the goal cylinder. We emphasize that in control experiments (1) and (2), from the periphery the animals reached the goal location that matches path integration through fairly direct as well as through tortuous routes, as in experiment Light.

To test whether the animals were influenced by the sight of the landmark array during the autonomous approach of the goal cylinder, we submitted the six subjects tested in the initial experiment (Light) to a second experiment (Dark). The (re)training and test procedures of experiment Dark were the same as those used in experiment Light, except that the full length of the trials occurred in darkness. Under these conditions (Table 1, lower part), the hamsters left the periphery to climb into a cylinder in only 24 out of a total number of 36 trials. One subject refused in all 6 trials (which were repeated twice at most), and two further subjects in 3 out of 6 trials, to proceed to the landmark array at the end of the guided outward

journey, returning instead to the nest. However, in the 24 trials that they did perform, the animals' first choice of a cylinder was wrong only once. The subjects therefore achieved 96% success.

In experiment Dark, systematic refusals to proceed towards the landmark array were more frequent than in experiment Light. Repeated refusals occurred in 8 test trials where the subjects had to cross the arena to reach the goal cylinder, and in 4 trials in which this was not the case. This suggests that the animals anticipated the distance they had to cover in darkness¹² and that the further they had to go to reach the goal, the more reluctant they were to leave the arena periphery. Moreover, to reach the goal from the arena periphery, the subjects tended to follow slightly more tortuous routes (Fig. 3) than in experiment Light. In particular, detours also occurred in those trials where the animals did not have to cross the arena.

To find the goal landmark at the end of the outward journey without seeing it, the animals must have learned to use non-visual cues, possibly derived from passive echolocation¹³. This is suggested by the fact that at the beginning of the training phase of experiment Dark, the subjects took the two training routes to the goal fairly directly until they had reached the approximate goal location, and then changed over to circular search movements¹⁴. These search movements disappeared in the course of the training phase of experiment Dark.

How did the animals plan their path to the goal cylinder in

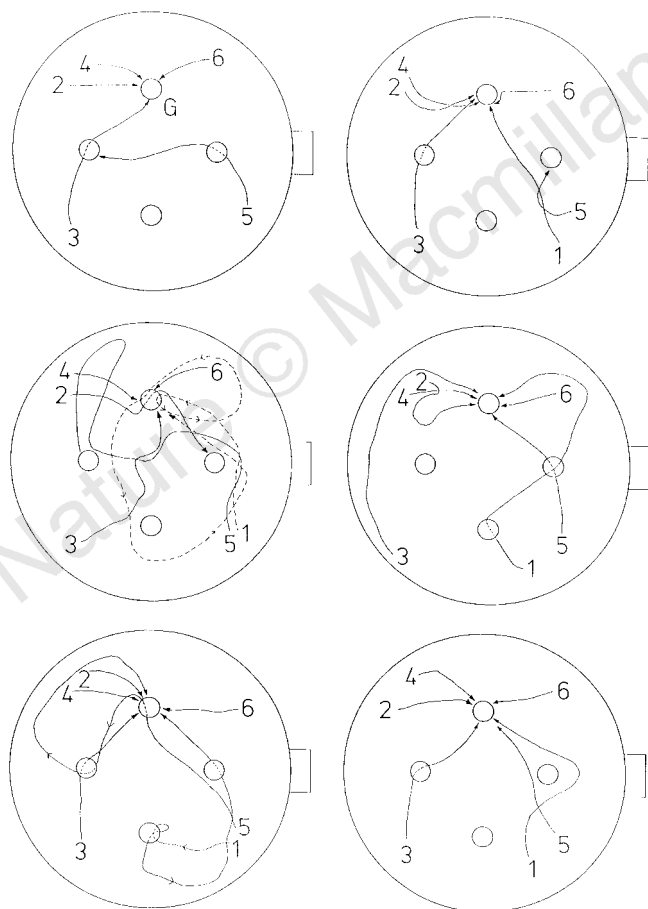


Figure 2 The autonomous paths followed by individual subjects in experiment Light. The circles represent the arena and the four cylinders, with the goal cylinder (G) always at the top of the cylinder array. In each circle, the continuous or broken lines with an arrow represent the paths followed by one subject from the arena periphery to the chosen cylinder. On its way to the goal, the animal sometimes passed under other cylinders. The number at the start of each path indicates the number of the corresponding trial (see top of Table 1). Each path was recorded from the moment the subject had clearly initiated its autonomous route to the goal.

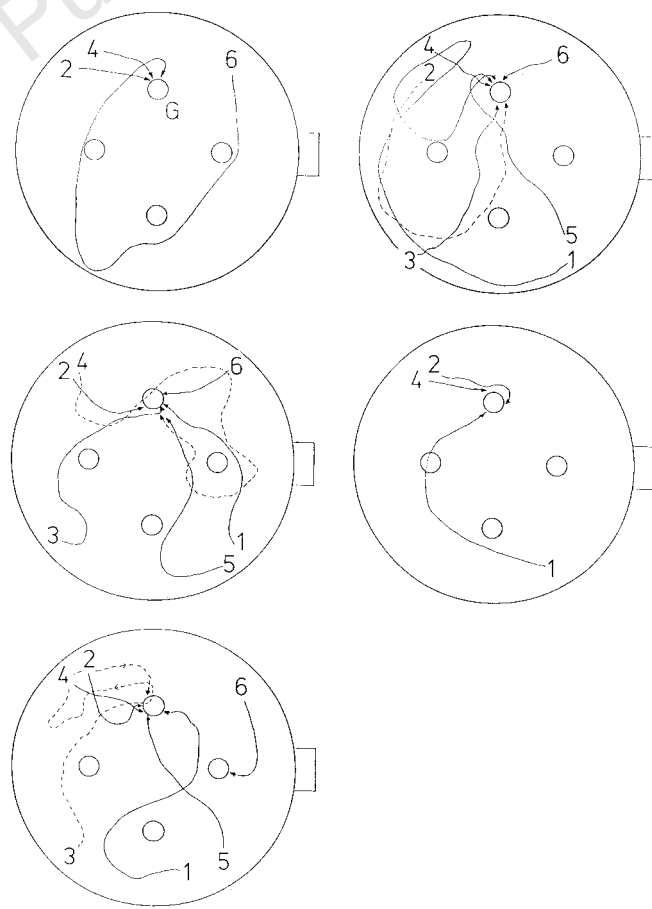


Figure 3 The autonomous paths followed by individual subjects in experiment Dark. The results from different individuals are presented in the same order as in Fig. 2. One subject refused in all trials to proceed to the landmark array. For further details, see Fig. 2 legend.

experiments Light and Dark? By proceeding along two different routes to the goal site during the preliminary training trials, the animals could learn the location of the goal with respect to the nest exit, and store this information in long-term memory as a nest-to-goal vector. Furthermore, path integration informs the animal continuously on its current position within the arena during a hoarding trip. To determine the final path to the goal cylinder, the animals had to combine the long-term nest-to-goal vector and the short-term current-position vector through vector addition. Thus, the hamsters may have subtracted the position vector from the goal vector at the moment they had to plan the autonomous path to the goal, at the end of the guided outward journey along the arena wall. According to a second hypothesis, basically the same computation may have gone on from the moment the animal left its nest, the animal subtracting continuously the path it actually followed from the stored goal vector. This process would constantly inform the animal on its position relative to its goal. But however the animals calculated the final path to the goal, the execution of the resulting goal vector again depended on path integration, the state of the path integrator informing the animal when it had reached the food goal. Note that in both experiments and in the controls the final path to the goal occurred along direct as well as circuitous routes: whereas path integration constantly informs the subject on the location of its goal, it does not constrain the shape of the goal-directed route.

The animals' basic reliance on self-generated vector information does not completely rule out the role of vision in experiment Light. Seeing the cylinder at the goal location may have confirmed vector information while the animal was still at the arena periphery and may therefore have encouraged the subject to walk towards the goal. On the other hand, the sight of four identical cylinders may also have confused the animals and therefore induced a relatively greater number of errors than when the hoarding trips took place in continuous darkness.

It has repeatedly been proposed that rodents plan the route from a new location to a familiar goal site through vector subtraction: the corresponding models imply that there is a combination between currently perceived and previously stored vectors that are linked to visual landmarks^{12,15,16}. We have shown that vector addition occurs independently of landmark constellations and therefore with respect to locations in absolute space. □

Methods

Apparatus. Each subject, always an adult female, lived during the experimental period in the test apparatus shown in Fig. 1, where it could commute through a small door¹¹ between an adjacent nest box and the open arena. Four identical cylinders formed a symmetrical landmark array around the arena centre. All cylinders contained an internal food cup 20 cm above ground level, but only the cylinder at location G was baited during training and test trials. The animal could enter the cylinders through four evenly spaced openings at their base to climb to the food cup. Subjects were filmed by an infrared camera above the arena centre: the arena was lit by infrared light¹¹ only (darkness) or additionally by four spotlights that were arranged symmetrically above the ceiling of the optical enclosure (light).

Procedures. In preliminary training trials, the subject followed a bait from the nest exit to one of two peripheral points (a or c); from these points, it learned to proceed to the cylinder at the goal location G, first by being led to G, then by walking autonomously towards it (Fig. 1). The guided part of the outward journey occurred first under light, then in darkness, the bait being dimly lit. The learning criterion was reached when the animal climbed into the cylinder at location G in at least 8 of 10 trials after having been led in darkness to point a or c. Before being submitted to a particular test trial, the subject had again to pass this criterion. In each of six different test trials, the subject was led in darkness via a new route along the arena periphery to point a, b, c or d. From each of these points it had to proceed alone towards the landmark array and climb into a cylinder. Trials in which the subject did not climb into a cylinder were repeated twice at most at the end of the respective experimental series. In experiment Light, the symmetrical lighting above the optical enclosure was

turned off shortly after the subject had entered the arena from its nest, and was turned on again when the animal started to proceed by itself from the arena periphery to the landmark array; the guided journey along the arena wall occurred in darkness. In experiment Dark, the entire (training and test) trials occurred in darkness.

Before each test session and each particular test trial, we neutralized potential olfactory (and tactile) cues within the arena by stirring, then flattening out the thick layer of sawdust on the arena floor. Further, we covered the arena walls with plastic bands 20 cm high, leaving only the exit door of the nest box open. Finally, the cylinders were systematically permuted, and so were the plastic bands.

Control experiments. Repeated attempts to test the role of uncontrolled external spatial cues in the relatively large standard arena failed, the animals refusing to leave the arena periphery at the end of the guided outward journey and to proceed to the landmark array. Knowing that the longer the required path, the less confident are the animals to navigate by path integration¹⁷, we tested five new subjects in a smaller arena (diameter, 160 cm). The distance from the arena centre to the symmetrical landmark array was proportionally the same as in the standard arena. In all control experiments, we submitted the subjects to the usual six test trials according to our usual test procedures. However, the cylinder at the goal location was never baited. (1) Control for room cues. Before the subject left its nest, we rotated the arena and nest by 90° with respect to their standard position. Each subject underwent two test series, the arena being rotated either clockwise or anticlockwise. (2) Control for both room cues and arena cues. Before the animal left the nest, we rotated the arena anticlockwise and the nest clockwise by 90° with respect to their standard position. (3) Control with an extended outward journey. We rotated the arena and nest by 90°, as in control experiment (1). In each of the six trials, we extended the guided outward journey by leading the subject from the next exit three times in a constant direction along the arena wall and then to the endpoint of the corresponding guided outward journey. As soon as the animal started the guided outward journey, the nest door was also covered by a plastic sheet. The whole guided outward journey occurred in darkness.

Recording and evaluation. The complete hoarding excursions were video filmed and then transcribed on a transparent paper on the video screen. The results were quantified in terms of the number of test trials in which the subjects climbed into a cylinder and the percentage of these trials in which the animal's first choice was the cylinder at the goal location. In the control trials, the number of correct choices was analysed by using the binomial test.

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Correspondence and requests for materials should be addressed to A.S.E. (Ariane.Etienne@pse.unige.ch) or R.M. (Roland.Maurer@pse.unige.ch).

State-dependent receptive-field restructuring in the visual cortex

Florentin Wörgötter, Katrin Suder, Yongqiang Zhao, Nicolas Kerscher, Ulf T. Eysel & Klaus Funke

Institut für Physiologie, Ruhr-Universität Bochum, D-44780 Bochum, Germany

To extract important information from the environment on a useful timescale, the visual system must be able to adapt rapidly to constantly changing scenes. This requires dynamic control of visual resolution, possibly at the level of the responses of single neurons. Individual cells in the visual cortex respond to light stimuli on particular locations (receptive fields) on the retina, and the structure of these receptive fields can change in different contexts^{1–4}. Here we show experimentally that the shape of receptive fields in the primary visual cortex of anaesthetized cats undergoes significant modifications, which are correlated with the general state of the brain as assessed by electroencephalography: receptive fields are wider during synchronized states and smaller during non-synchronized states. We also show that cortical receptive fields shrink over time when stimulated with flashing light spots. Finally, by using a network model we account for the changing size of the cortical receptive fields by dynamically rescaling the levels of excitation and inhibition in the visual thalamus and cortex. The observed dynamic changes in the sizes of the cortical receptive field could be a reflection of a process that

adapts the spatial resolution within the primary visual pathway to different states of excitability.

The frequency content of the electroencephalogram (EEG) is correlated to behavioural states. In a drowsy state, α -waves and some δ -waves predominate, whereas mainly β -waves are observed during attentive perception. This indicates that the temporal and spatial resolution of the visual system might also change in a way that is correlated with EEG frequency content. Indeed, the temporal characteristics of the cell responses in the visual thalamus (lateral geniculate nucleus, LGN) are markedly EEG-correlated. Brief stereotyped bursts of LGN neurons are seen during an α/δ -wave-dominated EEG (a synchronized EEG)^{5,6}, whereas the cells fire in a tonic, long-lasting way during a β -wave-dominated EEG (a non-synchronized EEG^{6–9}).

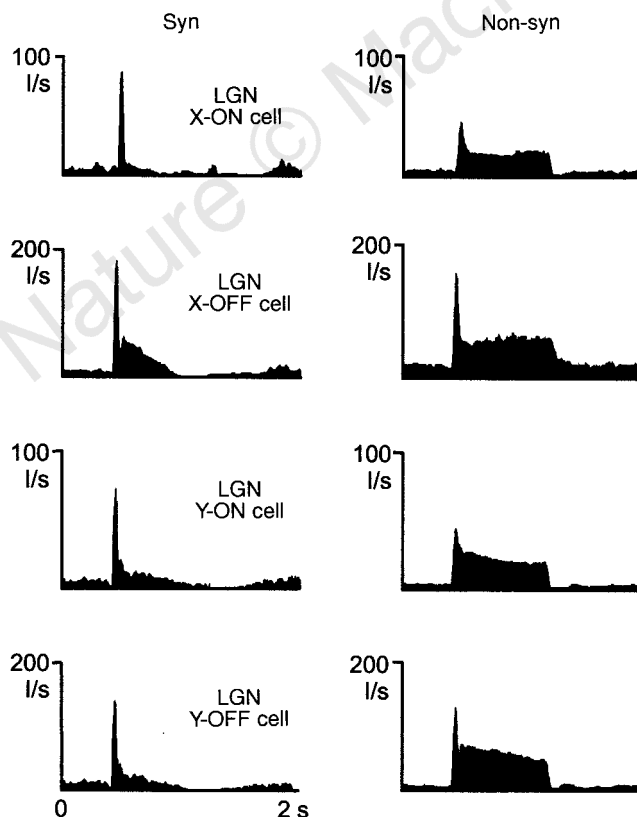


Figure 1 Temporal characteristics of LGN responses to flashing light spots (peristimulus time histograms) during different EEG states. Cells respond more tonically during non-synchronized EEG but more phasically during synchronized EEG. I/s, impulses per second. X and Y cells are different cell classes in the LGN.

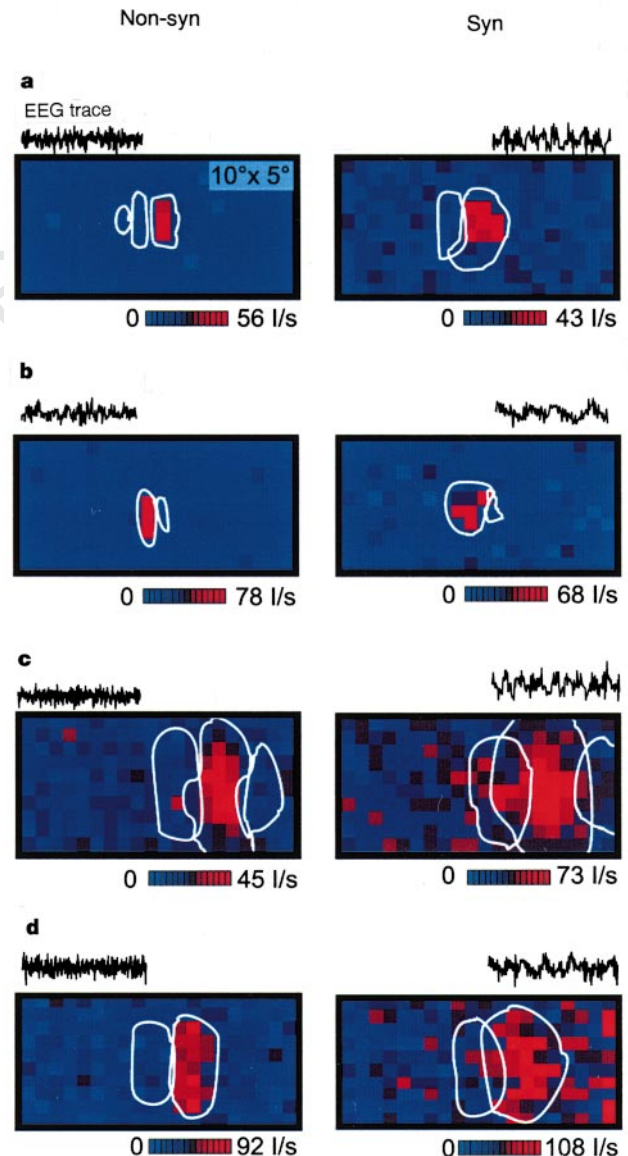


Figure 2 Changing shape of cortical receptive fields during different EEG states. The impulse rate of a single subfield is colour-coded according to the scales shown (maximum scaling) and the complete receptive field is outlined in white. Parts (5 s) from the corresponding EEG traces are shown. Receptive fields are wider during synchronized EEG and the subfield overlap increases. **a**, End-stopped simple cell 'Off' response; **b**, end-stopped simple cell 'On' response; **c**, simple cell 'Off' response; **d**, simple cell 'On' response. (End-stopped means that these cells are inhibited ('stopped') by stimuli which extend beyond the 'ends' of their receptive field.)

The peristimulus time histograms (PSTHs) shown in Fig. 1 were obtained from study of four LGN cells (total $n = 55$) during synchronized (left) and non-synchronized (right) EEG states. A strong and long-lasting tonic response component is visible only in the right-hand PSTHs. During synchronized EEG, however, the tonic responses are strongly diminished, whereas the phasic peaks have a high amplitude.

Neurons in the visual cortex are the main targets of LGN neurons. Therefore, one would expect that these two different temporal activity patterns of LGN cells should also have an effect on cortical cell behaviour. Figure 2 shows four representative receptive-field maps from a sample of 63 cortical cells recorded during the same EEG states as the PSTHs. Maps were obtained from cell responses, elicited with flashing bright and dark dot stimuli on a grid that covers the receptive field completely ('reverse-correlation method', flash duration 300 ms; this stimulus duration induces phasic and tonic LGN activity^{10,11}). Examples of individual 'On' or 'Off' subfields of simple cells are shown, recorded at different retinal eccentricities. The receptive field of each cell is substantially wider during a synchronized EEG (such that the subfield overlap increases) than during a non-synchronized EEG. The almost identical activity levels in the corresponding diagram pairs in Fig. 2 indicate that the observed receptive-field changes cannot be attributed merely to variations in overall activity which does not seem to be correlated to the EEG, as seen from the scale bars.

We determined quantitatively the degree of synchronization in the EEG and its correlation to the receptive-field size. The scatter plot in Fig. 3 shows that cortical receptive fields become significantly wider during synchronized EEG states. The receptive fields grow, on average, by 27% during an average 2.5-fold power increase in the δ -range (1–4 Hz). All cells were pooled, because very similar effects were found for simple ($n = 53$) and complex ($n = 10$) cells throughout all recording depths.

Another characteristic feature can be seen in the PSTHs in Fig. 1: the tonic response in an LGN cell during non-synchronized EEG is always preceded by a short phasic peak. We therefore predict that, during a non-synchronized EEG, a transition from a wider to a smaller receptive field should occur, in parallel with the transition from the phasic to the tonic part of the LGN response. We demonstrate this effect in Fig. 4, in which the width of the receptive

field is plotted against the time from stimulus onset to response of the cell (latency) for four more example cells. A fast, nonlinear shrinkage is seen after the first 50 ms of the response. This corresponds directly with the duration of the phasic LGN response, which is about 50 ms long (see PSTHs in Fig. 1) and rules out the possibility that normal adaptation effects, which have a duration of much more than 100 ms, are responsible for this effect.

These results confirm that cortex cells in general respond more vigorously to transient stimuli. However, this affects not only the temporal structure of their response but also the spatial shape of their receptive fields (Fig. 4). The percentage temporal change of the subfield width, comparing the sizes after 70 ms (100% value) with those after 200 ms, follows a distribution with a mean subfield shrinkage of $22.2\% \pm 12.6\%$. The median is 23.1% and the distribution is only slightly skewed (skewness +0.99); thus, the deviation from a gaussian distribution is small. The distribution yielded 54 out of 97 subfields, which shrank by more than 20%.

In a model we addressed the issue of which mechanisms might underlie the observed effects. We propose that temporal restructuring of the thalamic and cortical network activity leads to the observed effects (Fig. 5). The visual part of the thalamic reticular nucleus (the perigeniculate nucleus, PGN) integrates brain-stem activity, which is involved in the control of the sleep-waking cycle¹²,

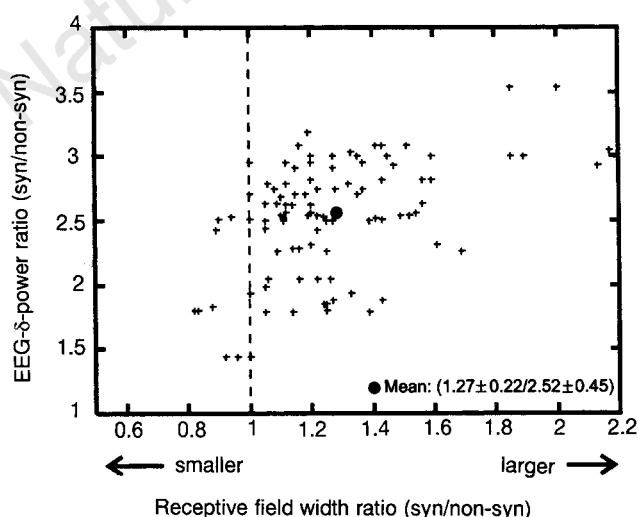


Figure 3 Scatter plot of EEG state versus subfield width for 97 subfields. The ratio of subfield width during synchronized EEG divided by that during non-synchronized EEG is plotted on the x-axis. The ratio of the power in the δ -range (1–4 Hz) for the same two recordings is plotted on the y-axis. The coordinate 1.0 indicates no change of the corresponding variable. Most samples (84 of 97) exhibit an increase in subfield size during increasingly synchronized EEG states.

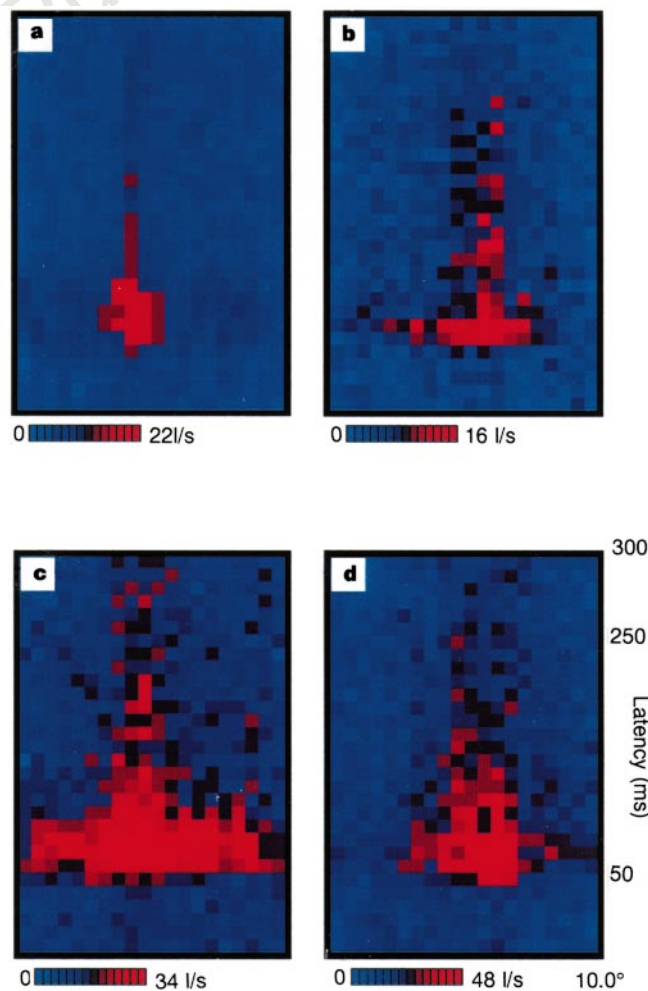


Figure 4 Shrinking of the receptive-field width for cortical cells as time after stimulation increases. Only the non-synchronized EEG state was studied. The data were analysed in successive windows of 10-ms duration. For every x-location in the cortical map, we collected the responses from all y-locations. **a**, End-stopped simple cell 'On' response; **b**, simple cell 'On' response; **c**, simple cell 'Off' response; **d**, complex cell 'On' response.

and thalamic relay cells are inhibited by cells of the PGN¹³. This inhibitory loop may be involved in the generation of different EEG waveforms such as sleep spindles¹⁴ and—together with the corticothalamic pathway—may act in the generation of synchronized δ -waves¹⁵.

The model indicates that a high level of inhibition of LGN neurons by PGN neurons during synchronized EEG may lead to bursting activity in the LGN, which, in turn, would result in wider and less specific cortical receptive fields. On the other hand, reduced LGN inhibition during non-synchronized EEG would drive the LGN cells into tonic transmission mode (often called 'single-spike mode'^{16,17}) and the cortical receptive fields would then be smaller (Fig. 5). Thus, the temporal structure of the thalamic activity could be a trigger that leads the subsequent intracortical interactions (for example, corticothalamic feedback and cortical lateral inhibition in the model) to react in a state-dependent way, resulting in the observed receptive-field restructuring.

The model predicts that cortical receptive fields should widen when the level of inhibition at the LGN cells is increased. This could be tested by, for example, microiontophoretic application of an excitatory transmitter in the PGN. The far-reaching PGN connec-

tions would inhibit a relatively large population of LGN cells, so that the predicted widening of receptive fields should be visible in the cortex.

Our experimental and modelling results indicate that a quite marked spatial restructuring of cortical receptive fields occurs during different EEG states, and that this effect could be due to the temporal structure of the thalamic cell (LGN and PGN) responses and the action of the corticothalamic and intracortical networks. These results indicate that temporal and spatial restructuring of visual cortical receptive fields may be important in controlling the resolution of visual processing in the primary visual pathway in a state-dependent way.

Methods

Experimental methods. Data were obtained from single-unit recordings, using glass pipettes in area 17 of four cats (63 cells total). Animals were first anaesthetized with ketamine. Surgery was performed to enable infusion of alcuronium chloride, Ringer solution and glucose through the femor artery and artificial respiration through the trachea ($N_2O : O_2 = 70 : 30$, 0.4% halothane) and to allow access to area 17. An additional craniotomy was made above area 18 to insert a 0.5-mm-diameter silver ball electrode epidurally for EEG recording. Blood pressure (>90 mm Hg) and end-expired CO_2 ($\approx 3.8\%$) were held within physiological limits. Cells were stimulated with small dots (0.5×0.5 degrees, $\pm 50\%$ contrast to background) on a computer screen. The screen was centred on the receptive field and rotated to the preferred orientation of the cell. The dot was presented about 200 times at every grid location in the diagram for 300 ms at each location (total recording time for a single cell >4 h). All spikes were counted in a window of 300-ms duration after a latency of 50 ms following every repeated stimulus presentation (reverse-correlation technique^{10,11}). See refs 18, 19 for more details.

Data analysis. We measured the relative power in the δ -range (1–4 Hz) with respect to the total power, up to 30 Hz¹⁸. A relative power in the δ -range of $>70\%$ was taken to represent highly synchronized EEG, and a relative power of $<30\%$ was taken to represent non-synchronized EEG; the middle range was not used. Finally, spike data were sorted with respect to these two ranges for presentation in the diagrams and statistical treatment. To determine the subfield width of a receptive field for every x -location in the map, we collected the responses from all y -locations. We then used the half-width at half-height, measured across the resulting peak, to characterize the width of a subfield.

Modelling. The model is restricted to a single 'On' subfield. Multiple antagonistic subfields can be generated by repeating the shown module. The same flashing stimulus as that used in the experiments was stimulated. Single model cells represented leaky 'integrate-and-fire' type neurons. LGN cells can switch between a depolarized, tonic transmission mode (maximum impulse rate ~ 250 Hz, contrast-dependent) and a hyperpolarized, phasic burst mode (2–8 spikes with interspike intervals of <2.5 ms, not contrast-dependent). To switch to the burst mode, PGN activity is increased; which is a major source of inhibition at the LGN cells^{20,21}. A PGN activity of 10 (or 25) spikes per second was used to mimic a non-synchronized (or synchronized) EEG state. Firing frequencies of PGN and LGN cells are approximately antagonistic¹⁹. We added synaptic noise at all stages. We simulated 4,000 cells on two two-dimensional grids (LGN/PGN and cortex) arranged topographically. Excitatory feed-forward connections are strong, whereas the corticothalamic feedback is weak and cannot drive the LGN cells on its own. The more tonically driven corticothalamic feedback enhances the depolarization level in the LGN only during non-synchronized EEG.

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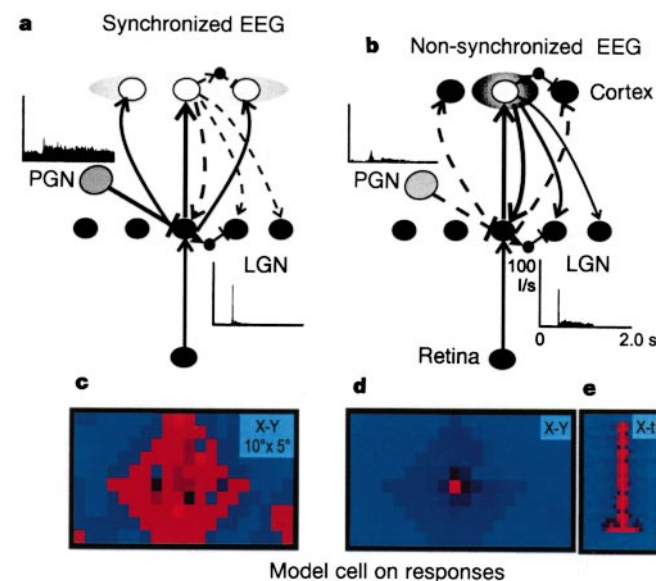


Figure 5 Model of the primary visual pathway during a synchronized and a non-synchronized EEG, and receptive-field maps for the corresponding states. **a**, Synchronized EEG; **b**, non-synchronized EEG; **c–e**, receptive-field maps. **a**, **b**, Insets show the activity of real LGN and PGN cells¹⁹. Anatomical connections²² are identical in both situations; different line thicknesses and types indicate only the effective connectivity²³, which changes in a state-dependent way. **a**, During synchronized EEG, inhibition of LGN neurons by PGN neurons is strong. LGN cells are, therefore, hyperpolarized⁵ and fire in phasic burst mode^{16,17}. The bursts of LGN activity are transmitted to the cortex. Because of the high firing frequency in a burst, strong temporal summation occurs at the target cells, and even those targets that receive weaker synapses (lateral to the main target cell group) become superthreshold. Wide cortical receptive fields are obtained. **b**, During non-synchronized EEG, inhibition by PGN neurons is weak. LGN cells are, therefore, depolarized⁵ and fire in tonic transmission mode^{16,17}. The lower firing frequencies in the sustained LGN activity result in a less marked temporal summation at their cortical targets and only cortical cells with strong synapses become activated (centre). This leads to the production of small cortical receptive fields. The prolonged cortical activity drives intracortical lateral inhibition and corticothalamic feedback. The excitatory feedback helps to keep the LGN cells in tonic transmission mode, while intracortical lateral inhibition further sharpens the cortical receptive field. **c**, Receptive field during a synchronized EEG state. **d**, Receptive field during a non-synchronized EEG state. **e**, Shrinking of the receptive-field width as time after stimulus increases. t , time.

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Correspondence and requests for materials should be addressed to F.W. (e-mail: worgott@neurop.ruhr-uni-bochum.de).

The nuclear hormone receptor SEX-1 is an X-chromosome signal that determines nematode sex

Ilil Carmi*, Jennifer B. Kopczynski† & Barbara J. Meyer*

* Howard Hughes Medical Institute and Department of Molecular and Cell Biology, University of California, Berkeley, Berkeley, California 94720-3204, USA

Organisms in many phyla determine sexual fate by distinguishing one X chromosome from two. Here we use the model organism *Caenorhabditis elegans* to dissect such an X-chromosome-counting mechanism in molecular detail. In this nematode, several genes on the X chromosome called X signal elements communicate X-chromosome dose by controlling the activity of the sex-determination gene *xol-1* (refs 1, 2). *xol-1* specifies male (XO) fate when active and hermaphrodite (XX) fate when inactive^{3,4}. The only X signal element described so far represses *xol-1* post-transcriptionally, but *xol-1* is repressed in XX animals by transcriptional and post-transcriptional mechanisms². Here we identify a nuclear-hormone-receptor homologue, SEX-1, that regulates the transcription of *xol-1*. We show that *sex-1* is vital to X-chromosome counting: changing *sex-1* gene dose in XX or XO embryos causes sexual transformation and death from inadequate dosage compensation (the hermaphrodite-specific process that equalizes X-gene expression between the sexes⁵). The SEX-1 protein acts directly on *xol-1*, associating with its promoter *in vivo* and repressing *xol-1* transcription in XX embryos. Thus, *xol-1* is the direct molecular target of the primary sex-determination signal, and the dose of a nuclear hormone receptor helps to communicate X-chromosome number to determine nematode sex.

To understand how small quantitative differences in molecular

signals can be translated into alternative developmental fates, we searched for components of the primary sex-determination signal in *C. elegans*. We conducted a genetic screen designed to detect regulators of *xol-1* (Fig. 1a). In the screen we used a *xol-1::lacZ* reporter transgene (*yls2*, Fig. 1b) that mimics the sex-specific response of the wild-type *xol-1* gene to the sex-determination signal, namely, high levels of activity in XO embryos and low levels in XX embryos⁴. We sought mutations that increased β -galactosidase activity in XX embryos, reasoning that such mutations might have reduced the X signal and thereby derepressed the endogenous *xol-1* gene. As *xol-1* also controls dosage compensation, derepression of *xol-1* in mutant XX animals causes death from increased X-gene expression⁴. Thus, to prevent hermaphrodite lethality from complicating the screen, we deleted *xol-1* from the parental strain. From 5,000 mutagenized haploid genomes, we identified two X-linked and 16 autosomal mutations. We show below that the X-linked mutation *y263* identifies an X-chromosome signal element, which we call *sex-1* (signal element on X).

As *yls2* encodes a translational fusion, our screen had the potential to identify both transcriptional and post-transcriptional regulators of *xol-1*. To distinguish between these possibilities, we studied the effect of *sex-1* mutations on a transcriptional *Pxol-1::lacZ* fusion, *yls33* (Fig. 1b). The original *sex-1* allele (*y263*), a second *sex-1* allele (*gm41*) obtained in an independent screen, and a chromosomal deficiency (*nDf19*) of the *sex-1* region all increased expression of *yls33* in XX embryos, indicating that *sex-1* may be a transcriptional repressor of *xol-1* in hermaphrodites and that *sex-1* mutations reduce or eliminate its function (Fig. 1c–e). High *Pxol-1::lacZ* expression in *sex-1* XX mutants was seen only during

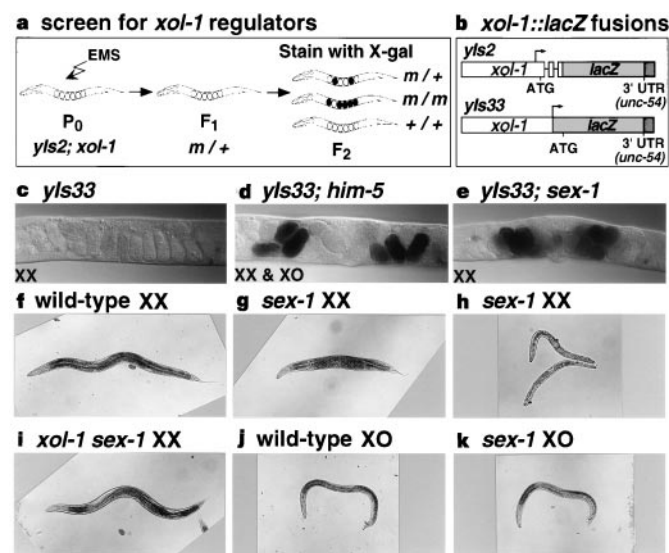


Figure 1 *sex-1* represses *xol-1* transcription in XX animals. **a**, The genetic screen used to identify regulators of *xol-1*, including *sex-1* (see Methods). *m* indicates a mutant gene. **b**, The integrated *xol-1::lacZ* translational⁴ (*yls2*) and *Pxol-1::lacZ* transcriptional² (*yls33*) reporter transgenes. Arrows denote presumed start points of transcription. UTR, untranslated region. **c–e**, Nomarski photomicrographs of gravid *yls33* hermaphrodites, fixed and stained with X-gal. **c**, Wild-type hermaphrodites produce only XX embryos, which express low levels of *lacZ*. **d**, *him-5* hermaphrodites produce ~33% XO embryos, which express high levels of *lacZ*, reflecting the XO-specific activation of *xol-1* transcription. **e**, *sex-1* mutations increase *lacZ* expression, suggesting that *sex-1* normally represses *xol-1* transcription in XX embryos. **f–k**, Photomicrographs of wild-type and *sex-1* mutant XX and XO animals. **f**, Wild-type hermaphrodite. **g, h**, The surviving *sex-1* XX mutants exhibit Dumpy and Egl phenotypes (**g**), which are characteristic of dosage-compensation defects, and masculinization (**h**), which is characteristic of sex-determination defects. **i**, *xol-1* mutations suppress all the *sex-1* mutant phenotypes. **j, k**, *sex-1* mutant males (**k**) are indistinguishable from wild-type males (**j**).

† Present address: Exelixis Pharmaceuticals, Inc., 260 Littlefield Avenue, South San Francisco, California 94080, USA

gastrulation, when peak expression of *xol-1* occurs in wild-type XO embryos, indicating that *sex-1* mutations disrupt only the sex specificity of *xol-1* transcription.

Is *sex-1* a repressor of *xol-1*, as predicted by the transgenic experiments, and, if so, is *sex-1* an X-chromosome signal element? An X signal element must exhibit the reciprocal dose effects in males and hermaphrodites that distinguish X elements from other regulators. Decreasing the copy number of X signal elements in hermaphrodites should derepress *xol-1* expression. The increased *xol-1* activity should promote male development and prevent activation of dosage compensation, thereby increasing X-gene expression and killing hermaphrodites. Conversely, increasing the copy number of signal elements in males should repress *xol-1*, promote hermaphrodite development and activate dosage compensation, thereby reducing X expression and killing males. Because the X-chromosome signal is multigenic, changing the dose of a single X element may only partly repress or derepress *xol-1*.

We assessed the effect of decreasing *sex-1* dose by studying the phenotype of homozygous *sex-1* XX mutants in a *xol-1*(+) background. The viability of *sex-1* homozygous mutant XX progeny from *sex-1* homozygous mutant mothers was reduced to 65% for *y263* and 37% for *gm41* homozygotes, compared with 101% viability of wild-type animals (Fig. 1f and Table 1a). XX survivors of lethality were dumpy (Dpy) and egg-laying-defective (Egl) (Fig. 1g), phenotypes characteristic of mutations that disrupt dosage compensation⁶. Also, ~20% of *sex-1* XX mutants were variably masculinized (Fig. 1h), revealing defects in sex determination. Both the dosage-compensation and the sex-determination phenotypes were suppressed by a *xol-1* null mutation (Fig. 1i and Table 1a), confirming that *sex-1* mutations derepress *xol-1* and that the lethality results from increased X-chromosome-gene expression. *sex-1* mutations had little or no effect on the viability of XO animals (Table 1c and Fig. 1j, k), indicating that they are XX-specific. In addition, *sex-1* mutations failed to suppress the XO-specific lethality caused by *xol-1* mutations, indicating that *sex-1* acts upstream of *xol-1* (results not shown). These results indicate that *sex-1* is a repressor of *xol-1* and are consistent with *sex-1* being an X signal element.

As sex is specified by the X-chromosome dose of the fertilized zygote, changes in the zygotic dose of *sex-1* alone should perturb *xol-1* expression, if *sex-1* is a signal element. In the experiments above, both maternal and zygotic doses of *sex-1* had been eliminated. To assess zygotic dose effects, we compared *sex-1* heterozygous and homozygous XX progeny from heterozygous mothers. All heterozygous XX progeny were viable and wild-type in morphology (results not shown). In contrast, 90% of the homozygous mutant progeny were viable (Table 1b), and all the mutants exhibited Dpy and Egl dosage-compensation phenotypes, which were suppressed by *xol-1* mutations (data not shown). Thus there is a zygotic *sex-1* activity that regulates *xol-1*. Furthermore, all heterozygous *sex-1* XX cross-progeny from homozygous mutant mothers were viable and wild-type in morphology (Table 1d), indicating that the zygotic contribution of *sex-1* in hermaphrodites is sufficient to repress *xol-1* and thereby determine sex.

Reciprocal experiments to determine whether increasing the dose of *sex-1*(+) kills XO males showed that two extra copies of *sex-1*, provided by the duplication *stDp2* (Fig. 2a), reduced male viability to 31%, compared with 78% viability of the duplication-bearing hermaphrodites (Table 2). This lethality results from changes in the zygotic dose of *sex-1*, as the viability of males with two copies of *stDp2* was the same (30%) whether the mother carried one or two copies of *stDp2* (Table 2). A weak *sdc-2* mutation that disrupts dosage compensation rescued the duplication-bearing males, showing that the lethality was caused by reduced X-gene expression: 70% of the *stDp2/stDp2; sdc-2* males were viable, a level comparable to hermaphrodite viability in the same genetic background (Table 2). More importantly, *stDp2/stDp2* males were also rescued by *sex-1* mutations (70% viability, Table 2), indicating that the male-specific lethality was caused by an increase in *sex-1* dose and not an increase in the dose of other genes covered by the duplication. In summary, changes in the zygotic dose of *sex-1* cause the reciprocal effects on males and hermaphrodites expected of a counted X signal element that specifies sexual fate.

Genetic interactions between *sex-1* and the gene encoding the putative RNA-binding protein FOX-1 (refs 7, 8), an X signal element that regulates *xol-1* post-transcriptionally⁷, demonstrate

Table 1 *sex-1* mutations cause hermaphrodite-specific lethality

(a) Maternal genotype	Genotype of XX progeny	Viability of XX progeny*	No. of adults/Total embryos
<i>sex-1</i> (+)	<i>sex-1</i> (+)	101%	1,254/1,246
<i>sex-1</i> (<i>y263</i>)	<i>sex-1</i> (<i>y263</i>)	65%	748/1,150
<i>sex-1</i> (<i>gm41</i>)	<i>sex-1</i> (<i>gm41</i>)	37%	233/627
<i>xol-1</i> (<i>y9</i>) <i>sex-1</i> (<i>y263</i>)	<i>xol-1</i> (<i>y9</i>) <i>sex-1</i> (<i>y263</i>)	93%	3,614/3,883
(b) Maternal genotype†	Genotype of XX progeny	Viability of <i>sex-1</i> (+) or <i>sex-1</i> (-) XX progeny‡	No. of <i>sex-1</i> (+) or <i>sex-1</i> (-) adults/Total other progeny§
<i>sex-1</i> (+) <i>m</i> ₁	<i>sex-1</i> (+)	100%	476/1,420
<i>sex-1</i> (<i>y263</i>) <i>m</i> ₁	<i>sex-1</i> (<i>y263</i>)	90%	510/1,746
<i>sex-1</i> (<i>gm41</i>) <i>m</i> ₁	<i>sex-1</i> (<i>gm41</i>)	90%	519/1,810
(c) Maternal genotype¶	XO viability¶¶	No. of XO adults/No. of XX adults	
<i>m</i> ₂ <i>sex-1</i> (<i>y263</i>)	101%	1,353/1,343	
<i>m</i> ₂ <i>sex-1</i> (<i>gm41</i>)	89%	1,028/1,156	
(d) Maternal genotype#	Viability of <i>sex-1</i> (+) XX cross-progeny*	No. of <i>sex-1</i> (+) XX progeny/Total embryos	
<i>m</i> ₃ <i>sex-1</i> (<i>y263</i>)	100%	633/630	
<i>m</i> ₃ <i>sex-1</i> (<i>gm41</i>)	101%	302/299	

* Viability of the homozygous XX progeny was calculated as the ratio of live adults to total counted embryos laid by mothers of the specified genotype.

† *m*₁ denotes the dominant marker *unc-58*, which is closely linked to *sex-1* (Fig. 2b). The enhanced lethality of *sex-1* homozygous mutants from *sex-1* homozygous mothers compared with *sex-1* heterozygous mothers need not indicate a role for *sex-1* beyond that of repressing *xol-1*, for three reasons: *xol-1* suppresses all *sex-1* lethality; *sex-1* mutant males appear wild-type; and the *sex-1* mutant phenotype can be rescued zygotically.

‡ Viability was calculated as the ratio of the number of non-Unc (*sex-1* mutant) animals observed to the number expected based on the number of Unc (*sex-1/unc-58* or *unc-58/unc-58*) progeny. As *unc-58* is dominant, 25% of the progeny are expected to be non-Unc. All of these homozygous *sex-1* XX mutants show Dpy and Egl phenotypes.

§ The numbers indicate the ratio of non-Unc progeny to Unc progeny produced by mothers of the indicated genotype.

¶ *m*₂ denotes the X-linked markers *unc-1* or *unc-2* (Fig. 2a).

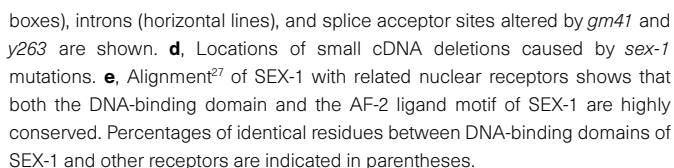
¶¶ Wild-type (N2) males were mated with marked *sex-1* mutant hermaphrodites of the indicated genotype and the numbers of cross-progeny males (*m*₂*sex-1*) and hermaphrodites (*m*₂*sex-1* / +) were determined. As equal numbers of males and hermaphrodites are expected, viability was calculated as the ratio of live males to live hermaphrodites.

*m*₃ denotes the X-linked marker *unc-2*.

* *tra-2*(*q276*) XX males were mated with hermaphrodites of the indicated genotype. The total number of embryos laid by these hermaphrodites and the numbers of cross-progeny (non-Unc) and self-progeny (Unc) were determined. The parents were transferred to fresh plates every day, and only plates on which self-progeny hermaphrodites constituted <5% of the total progeny were included in the final calculation. Viability was calculated as the ratio of live cross-progeny to the number of embryos laid minus the number of self-progeny.

Increasing the dose of *sex-1* kills males by activating the hermaphrodite mode of dosage compensation.
 * Number of wild-type *sex-1* copies present in XO animals of the genotype scored.
 † Viability was calculated as the ratio of adult males or hermaphrodites to the expected number of adults of each sex. The number of males expected is $0.32 \times 0.79 \times$ number of embryos. The number of hermaphrodites expected is $0.68 \times 0.79 \times$ number of embryos. The number of expected males and hermaphrodites is dictated by the proportions of each sex observed in *him-5* strains: 32% of the progeny of *him-5* hermaphrodites are male (360 males per 1,144 total adults). The factor 0.79 was used because only 79% of *him-5* mutants are viable (1,144 adults/1,450 total embryos).
 ‡ Number of embryos laid by mothers of the indicated genotype.
 § Genotype of counted males and hermaphrodites. Unless otherwise noted, maternal genotype was identical. *stDp2* is a translocation of X chromosome genes to chromosome II (ref. 26).
 || Maternal genotype was *stDp2/rol-6(su1006d); him-5; dpy-6*. Homozygous *stDp2/stDp2* hermaphrodite and male progeny were identified by their wild-type (non-Rol) phenotype.
 ¶ Viability was calculated as above, except that the number expected was multiplied by 0.25 because only a quarter of the progeny are expected to have two copies of *stDp2*.
 #y93 is a weak *sdc-2* allele that causes little or no XX lethality (1,606 adults/1,658 embryos or 97% viability).

The rescuing ORF corresponds to a previously identified complementary DNA⁹ that encodes a 555-amino-acid protein (named CNR14) with high similarity to members of the nuclear-hormone-receptor (NHR) superfamily¹⁰. NHRs possess highly conserved DNA- and ligand-binding domains. The SEX-1 DNA-binding domain shares 60–80% identity with other NHR-family members (Fig. 2e), suggesting conservation of DNA-binding ability. In addition, SEX-1 contains an activation function-2 (AF-2) motif (Fig. 2e), a sequence required for ligand-dependent activation. However, the remainder of its ligand-binding domain (LBD) has diverged from other characterized LBDs^{11–14}, making it difficult to predict



whether SEX-1 uses a ligand. The closest SEX-1 homologues are E78A, a *Drosophila* ecdysone-inducible protein of unknown function¹⁵, and Rev-Erb, an orphan receptor that functions as a transcriptional repressor *in vitro*¹⁶. Thus, the molecular similarity of SEX-1 to NHRs is consistent with its role as a transcriptional repressor of *xol-1*.

DNA-sequence analysis showed that both *sex-1* mutant alleles create changes within the coding region, confirming the identity of the gene. Both *y263* and *gm41* cause G → A transitions that alter splice acceptor sites at the junctions of intron 4 and exon 5, and intron 5 and exon 6, respectively, downstream of the DNA-binding domain (Fig. 2c). cDNA sequence analysis showed that these mutations cause incorrect splicing (Fig. 2d). In *y263*, a 17-base-pair deletion of coding sequence introduces a frameshift that truncates the protein at amino acid 233. In *gm41*, a 2-nucleotide deletion induces a frameshift that truncates the protein at amino acid 288. Both mutant proteins are predicted to retain the DNA-binding domain.

To determine the expression pattern and cellular localization of

SEX-1, we raised antibodies against the first 152 amino acids of SEX-1 and analysed the antibody-staining pattern in mutant and wild-type embryos. Staining was not detected in homozygous *y263* or *gm41* mutant embryos (Fig. 3d–f), confirming the specificity of SEX-1 antibodies and the reduction of gene function in *sex-1* mutants. In wild-type animals, ubiquitous nuclear staining (Fig. 3a–c) was observed from oogenesis through to midembryogenesis. SEX-1 expression peaked at the onset of gastrulation (at about the 28–100-cell stage), then gradually diminished, becoming undetectable by the end of embryonic cell proliferation (the 550-cell stage) (results not shown). SEX-1 appeared to be expressed similarly in XO animals. The timing and localization of SEX-1 are consistent with a role for *sex-1* as a signal element and indicate that SEX-1 could act directly on *xol-1* to repress its transcription. *xol-1* transcription is required from the 28-cell stage to the end of gastrulation (~380-cell stage) to trigger male development and must be repressed in XX embryos during that time to allow hermaphrodite development.

Further evidence supports the idea that SEX-1 represses *xol-1*

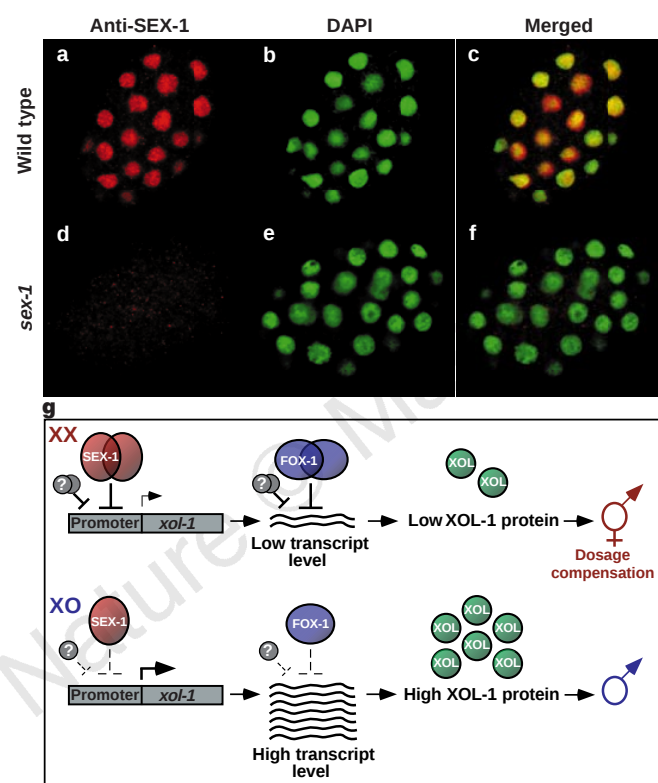


Figure 3 The temporal and spatial expression of SEX-1 are consistent with its role as an X signal element that represses *xol-1* transcription. **a–f**, False-colour confocal images of wild-type (**a–c**) and *sex-1*(*y263*) (**d–f**) embryos stained with anti-SEX-1 antibodies (red) and the DNA-intercalating dye 4,6-diamidino-2-phenylindole (DAPI) (green). SEX-1 protein is localized to nuclei of wild-type XX embryos, but is absent from nuclei of *sex-1* mutant embryos and wild-type embryos that have completed cell proliferation. **g**, A model for regulation of *xol-1*. In XX animals the cumulative dose of several X signal elements represses *xol-1* activity. The nuclear hormone receptor SEX-1 represses *xol-1* transcriptionally, whereas the RNA-binding protein FOX-1 represses *xol-1* post-transcriptionally². Other X signal elements (grey spheres), whose presence is inferred through analysis of X-chromosome deletions and duplications¹, also control *xol-1* using both mechanisms². The two forms of repression act together to establish low XOL-1 protein levels in XX animals, thereby allowing hermaphrodite development, including dosage compensation. In XO animals, the single dose of signal elements allows high XOL-1 protein levels to accumulate, leading to male development, including repression of dosage compensation.

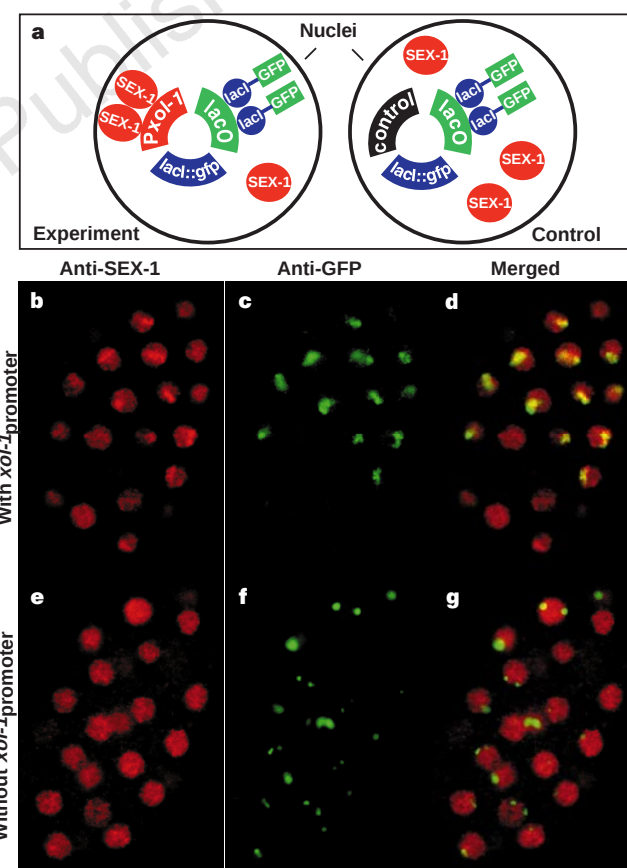


Figure 4 SEX-1 localizes to extrachromosomal DNA arrays containing *xol-1* promoter sequences. **a**, The array-binding experiments. The *lacI::GFP* fusion protein (blue circle and green rectangle) binds to *lacO* sequences, allowing visualization of both experimental and control arrays with anti-GFP antibodies. If the endogenous SEX-1 protein (red ovals) associates with *xol-1* promoter sequences, anti-SEX-1 antibodies should co-localize with anti-GFP antibodies on arrays containing the *xol-1* promoter (left) but not on arrays lacking the promoter (right). **b–g**, Confocal images of wild-type embryos containing *xol-1* promoter arrays (**b–d**) or control arrays (**e–g**). SEX-1 (red) and *lacI::GFP* (green) co-localize (yellow) on arrays containing *xol-1* promoter DNA, indicating that the sex-determination gene *xol-1* is the direct target of the nuclear hormone receptor SEX-1 *in vivo*.

directly. The integrated *yIs33* array, which contains multiple copies of the *Pxol-1::lacZ* transgene, strongly suppressed the male lethality caused by two copies of *stDp2* (Table 2), implying that extra copies of the *xol-1* promoter may titrate SEX-1 and thereby prevent the endogenous *xol-1* gene from being repressed effectively.

To prove that *xol-1* is the direct molecular target of SEX-1, we devised a strategy to assess whether SEX-1 associates with *xol-1* promoter sequences *in vivo* (Fig. 4a). We created transgenic strains containing extrachromosomal DNA arrays with multiple copies of either the *xol-1* promoter or control DNA. These arrays also carried multiple copies of the *lac* operator (*lacO*) and a transgene encoding the *lac* repressor tagged with green fluorescent protein (*lacI::GFP*). The tagged *lac* repressor binds to the *lac* operator sequences within the extrachromosomal array, allowing the array to be detected by anti-GFP antibodies. If the endogenous SEX-1 protein binds directly to the *xol-1* promoter, anti-SEX-1 antibodies should co-localize with anti-GFP antibodies on arrays containing the *xol-1* promoter but not on arrays lacking the promoter (Fig. 4a). Indeed, anti-SEX-1 antibodies consistently recognized arrays containing the *xol-1* promoter (Fig. 4b–d) and rarely recognized control arrays lacking the *xol-1* promoter (Fig. 4e–g). Less than 10% of control embryos exhibited infrequent (<10% of arrays) co-localization, whereas more than 60% of experimental embryos exhibited frequent (>80% of arrays) co-localization, thus showing that SEX-1 associates with *xol-1* promoter sequences *in vivo* to repress *xol-1* transcription in XX animals. These results are the first molecular demonstration that *xol-1* is the direct molecular target of the primary sex-determination signal, thus proving that *xol-1* counts X-chromosome dose.

As a *xol-1* repressor, SEX-1 may function in one of at least two ways. The similarity of SEX-1 to the orphan receptor Rev-Erb and the lack of a conserved ligand-binding domain in SEX-1 indicate that SEX-1 may act as an unliganded constitutive transcriptional repressor. However, SEX-1, unlike Rev-Erb, contains an AF-2 motif, which may indicate ligand-binding ability^{11–14}. Thus, an alternative model is that SEX-1 acts as a ligand-dependent repressor. This mode of repression has been demonstrated in cell lines for the inhibition of AP1-mediated transcriptional activation by the retinoic-acid and cErbA receptors¹⁷. This second model suggests that cell–cell interactions might contribute to the initial sex-determination decision in *C. elegans*, perhaps to ensure that all cells in the animal adopt the same sexual fate.

Coincidentally, NHRs distantly related to SEX-1 have been implicated in mammalian sex determination. The human NHR gene *DAX1* maps to a region on the X chromosome whose duplication causes sex reversal of XY males¹⁸. In addition, extra copies of the murine *Dax1* homologue cause sex reversal of male mice¹⁹. Whether *Dax1* acts in wild-type XX mammals as a sex signal to initiate female development, as SEX-1 does in worms, is not yet known. However, *Dax1* and SEX-1 dosage studies indicate that molecular parallels previously proposed for sexual differentiation²⁰ in *C. elegans* and mammals may extend further to the earliest signalling steps of sex determination, implying at the very least a convergence of molecular strategies. □

Methods

Genetic screen for *xol-1* regulators. Hermaphrodites of genotype *yIs2; xol-1(y9)* were mutagenized with ethyl methanesulphonate (EMS)²¹, their F₁ progeny were picked to individual plates, and 30 gravid F₂ hermaphrodites from each F₁ progeny were stained with X-gal²² to identify XX mutants with increased *lacZ* expression. Mutant lines with more than 1 blue embryo per hermaphrodite were recovered from sibling F₂ animals.

sex-1(gm41) was identified by G. Garriga in a screen for mutants with abnormal hermaphrodite-specific neuron (HSN) migration and provided by P. Baum, who recognized the sex-determination defects.

Genetic mapping of *sex-1*. Chromosomal linkage of *sex-1(y263)* was determined by scoring the segregation of genetic markers relative to blue XX

embryos, revealing linkage between *y263* and *dpy-6* on the X chromosome. Both *sex-1* mutant alleles were mapped relative to X-linked sequence-tagged site (STS) polymorphisms in the RW7000 strain²³. From *sex-1/RW7000* heterozygotes, ten *sex-1* recombinants had *stP103* and STS markers to the left; two recombinants had *stP129* and markers to the right, placing *sex-1* between *stP103* and *stP129*. Further mapping was achieved by picking Dpy non-Unc and Unc non-Dpy (where Unc is uncoordinated) recombinants from *y263/dpy-6 unc-115(e2225)* heterozygotes and scoring for the *sex-1* mutant phenotype. None of 54 Unc non-Dpy recombinants and 11 of 14 Dpy non-Unc recombinants showed the *sex-1* mutant phenotype. Three of the fourteen recombinants could not be scored. These data placed *sex-1* to the right, or close to the left, of *unc-115*.

A polymerase chain reaction (PCR)-based assay showed that *nDf19* removes *sex-1*. Individual *nDf19/nDf19* embryos from *unc-76(e911); nDf19/lon-2* mothers did not amplify a *sex-1*-specific band in PCRs but did amplify a control band from *sdc-3*. *y263/nDf19* hermaphrodites are Dpy and Egl, and their viability is slightly reduced relative to viability of *y263* homozygotes. However, not all *nDf19/+* heterozygotes are viable and others are sick, making it plausible that the enhanced lethality of *y263/nDf19* mutants compared with that of *y263/y263* animals does not result from further reduction of *sex-1* activity.

***fox-1 sex-1* double mutant.** Although *fox-1* and *sex-1* are >13 map units apart, attempts to recover a *fox-1 sex-1* double mutant from 16 *fox-1 unc-2/sex-1* heterozygotes yielded only a few (<10) very dumpy sterile hermaphrodites, indicating that the double mutants were dead. Lethality of this XX double mutant was verified through analysis of the *fox-1 unc-2 sex-1/szT1* strain, which produced *fox-1 unc-2 sex-1* males (Unc) but not hermaphrodites.

Physical mapping of *sex-1*. *sex-1* was mapped relative to four restriction-fragment length polymorphisms (RFLPs), identified between RW7000 and N2, namely *yP4*(CO8D1), *yP5*(RO7E3), *yP6*(KO8A5) and *yP7*(WO5A12) (Fig. 2). From *unc-115 sex-1/RW7000* heterozygotes, we picked Unc non-Dpy and Dpy non-Unc recombinants and probed genomic DNA for RFLPs. Three of three Unc non-Dpy recombinants carried *yP5*, *yP6* and *yP7*, whereas none of three Unc non-Dpy recombinants carried *yP4*. The single Dpy non-Unc recombinant contained *yP4* but not *yP5*, *yP6* or *yP7*. These results indicate that *sex-1* is located between *yP4* and *yP5*.

Transformation rescue of *sex-1* mutants. Cosmids (5–40 µg ml⁻¹) from the *sex-1* region were injected with the dominant marker *rol-6* (pRF4, 100 µg ml⁻¹) into the syncytial gonad of young *sex-1(gm41)/unc-115* mutant hermaphrodites to test for rescue. Rescue was scored as the absence of Dpy Rol hermaphrodite progeny from the injected hermaphrodites. Three of three lines containing the cosmid CO6F4 and three of four lines carrying the overlapping cosmid F44A6 rescued *sex-1*. The ORF responsible for rescue was identified by injecting individual subclones containing each shared ORF. Two of seven lines containing the F44A6.2 ORF (10,409-base-pair *SmaI/HindIII* fragment) rescued *sex-1* mutant hermaphrodites; four lines gave partial rescue. To achieve rescue, we injected this fragment at a low concentration (5 µg ml⁻¹), because higher concentrations caused lethality in otherwise wild-type males and hermaphrodites. The minimal rescuing region was defined by deleting a *SpeI* fragment, leaving F44A6.2 and 1,463 base pairs upstream of the first ATG codon. One of three lines containing this clone rescued *sex-1* mutants.

DNA-sequence analysis. Random-primed cDNA made from RNA of *y263*, *gm41* and N2 strains was selectively amplified in PCRs using six *sex-1* primer sets to create overlapping DNAs. DNA-sequence analysis of three independent PCR products from each primer set revealed a deletion of nucleotides 659–675 from *y263* and nucleotides 856–857 from *gm41*. Genomic DNA sequencing revealed only a G → A transition at nucleotide 2,694 in *y263* and a G → A transition at nucleotide 2,946 in *gm41*.

SEX-1 antibodies. Rabbit polyclonal antibodies were generated against a His₆-tagged fragment of SEX-1 that included amino acids 1–152. This protein was expressed and purified according to Qiagen protocols. Anti-SEX-1 antibodies were affinity-purified over a column of Affi-Gel 15 beads (Bio-Rad) coupled to a fusion protein containing maltose-binding protein and SEX-1 amino acids 1–152. Embryos were fixed and stained as described²⁴.

***xol-1* promoter binding.** To create array-bearing strains, we injected wild-type hermaphrodites with four plasmid DNAs. One plasmid contained the *xol-1* promoter as in *yIs33* (–2,850 to –75 base pairs in pBluescript, pTY1488); the

second contained tandem repeats of the *lac* operator (pSV2-dhfr; ref. 25); the third contained *lacI::gfp* driven by the heat-shock promoter *hsp16-2* (pPD49-78; A. Gonzalez-Serrichio and P. Sternberg, manuscript in preparation); and the fourth contained the dominant marker *rol-6*(pRF4), all at 50 $\mu\text{g ml}^{-1}$. Control arrays lacking *Pxol-1* were made from a similar DNA mix, with pBluescript replacing pTY1488. We studied embryos from five strains with independent *Pxol-1* arrays and two strains with independent control arrays. To obtain embryos, L4 roller hermaphrodites were picked onto plates and allowed to reproduce for 3–4 days at 20 °C. When F₁ hermaphrodite progeny became gravid, plates were shifted to 37 °C for 30 min to heat-shock the animals and induce expression of *lacI::gfp*. Once animals had recovered for 30 min at room temperature, embryos were collected, fixed and stained as above. GFP was detected using the anti-GFP monoclonal antibody 3E6 (Quantum Biotechnologies). At least 100 array-bearing embryos of experimental and control classes were studied by epifluorescence and 25 embryos of each array class were studied by confocal microscopy.

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Correspondence and requests for materials should be addressed to B.J.M. (e-mail: bjmyer@uclink4.berkeley.edu). The Genbank accession number for the *sex-1* sequence is AF049869.

Late embryonic lethality and impaired *V(D)J* recombination in mice lacking DNA ligase IV

Karen M. Frank^{*†‡}, JoAnn M. Sekiguchi^{*†‡}, Katherine J. Seidl^{*†§}, Wojciech Swat^{*†||}, Gary A. Rathbun^{†||}, Hwei-Ling Cheng^{*§}, Laurie Davidson^{*§}, Landy Kangaloo^{*§} & Frederick W. Alt^{*†||}

^{*} The Children's Hospital, [†] Department of Genetics, [§] Howard Hughes Medical Institute and ^{||} The Center for Blood Research, Harvard University Medical School, Boston, Massachusetts 02115, USA

[‡] These authors contributed equally to this work

The DNA-end-joining reactions used for repair of double-strand breaks in DNA and for *V(D)J* recombination, the process by which immunoglobulin and T-cell antigen-receptor genes are assembled from multiple gene segments, use common factors. These factors include components of DNA-dependent protein kinase (DNA-PK), namely DNA-PKcs and the Ku heterodimer, Ku70–Ku80, and XRCC4 (ref. 1). The precise function of XRCC4 is unknown, but it interacts with DNA ligase IV. Ligase IV is one of the three known mammalian DNA ligases²; however, the *in vivo* functions of these ligases have not been determined unequivocally. Here we show that inactivation of the ligase IV gene in mice leads to late embryonic lethality. Lymphopoiesis in these mice is blocked and *V(D)J* joining does not occur. Ligase IV-deficient embryonic fibroblasts also show marked sensitivity to ionizing radiation, growth defects and premature senescence. All of these phenotypic characteristics, except embryonic lethality, resemble those associated with Ku70 and Ku80 deficiencies^{3–6}, indicating that they may result from an impaired end-joining process that involves both Ku subunits and ligase IV. However, Ku-deficient mice are viable, so ligase IV must also be required for processes and/or in cell types in which Ku is dispensable.

The lymphoid-specific recombination-activating-gene proteins 1 and 2 (RAG 1 and 2) initiate *V(D)J* recombination by cleaving DNA at conserved recombination signal sequences (RSSs) that flank all antigen-receptor variable-region gene segments, generating coding and RSS ends. RSS ends are then precisely ligated, whereas coding ends are joined in a process that can involve nucleotide loss or gain in addition to ligation (see ref. 7 for a review). The joining phase requires proteins such as DNA-PKcs, Ku70/Ku80 and XRCC4 (ref. 1). Mice lacking Ku70 and Ku80 are immunodeficient because of an inability to rejoin coding or RSS ends^{3–6}, whereas DNA-PKcs-deficient mice are immunodeficient because of defects in rejoining coding, but not RSS, ends^{8,9}. These and other differences between Ku70/Ku80- and DNA-PKcs-deficient mice indicate that Ku proteins may have some DNA-PKcs-independent functions. The XRCC4 gene was isolated on the basis of its ability to complement the defect in joining coding and RSS ends of the ionizing-radiation sensitive XR-1 mutant cell line¹⁰. XRCC4, a nuclear protein that forms homodimers¹¹, may be involved in a late *V(D)J* recombination step such as ligation^{10,12}, and it associates with DNA ligase IV *in vivo*^{13,14} and stimulates its activity *in vitro*¹³. Mammalian cells contain at least two other DNA ligases, DNA ligases I and II/III (ref. 2). DNA ligase I probably functions during DNA replication and DNA ligases I and II/III in recombination and repair. There is little information about the *in vivo* role of mammalian DNA ligase IV (refs 13–16), but studies of *Saccharomyces cerevisiae* mutants indicated that the yeast homologues of Ku, ligase IV and XRCC4 are involved in a common DNA-end-joining pathway^{17–20}, further supporting a role for ligase IV in *V(D)J* recombination. In contrast, studies of an *in vitro* reaction that aimed to represent *V(D)J* recombination showed that DNA ligase I alone may be important in this process²¹.

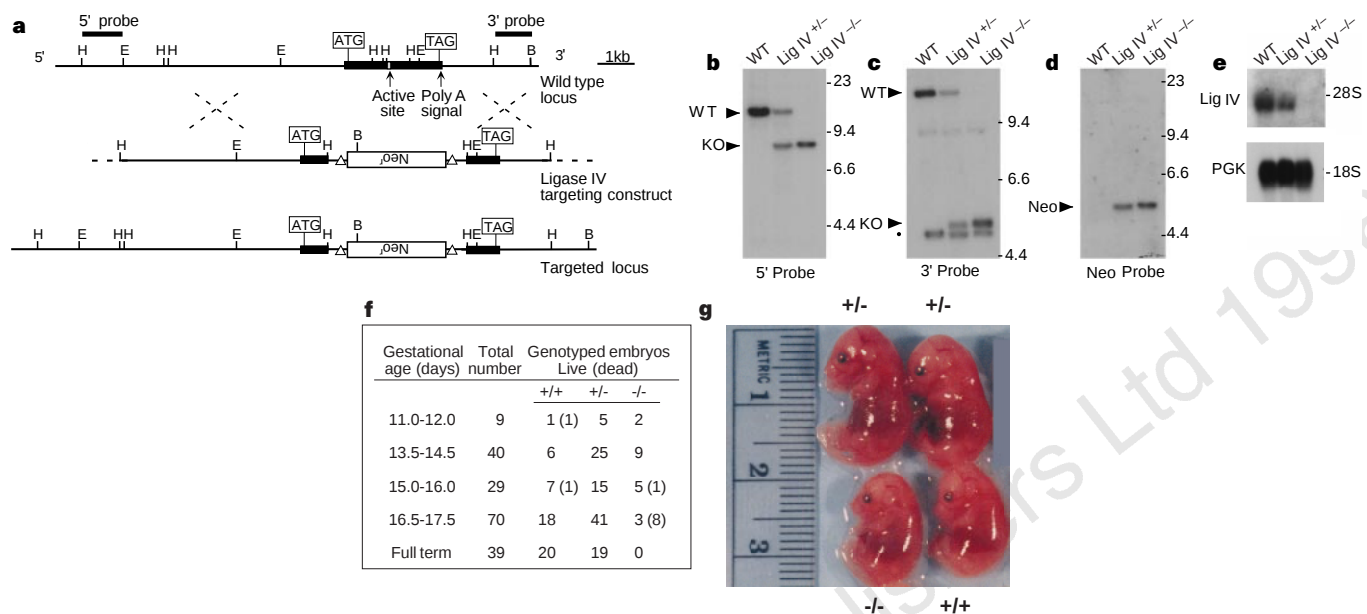


Figure 1 Targeting of the DNA ligase IV gene. **a**, Top, genomic locus of DNA ligase IV. Middle, targeting vector designed to insert a *neo*^r cassette into the ligase IV exon. Bottom, targeted locus. Restriction enzymes: H, *Hind*III; E, *Eco*RI; B, *Bam*HI. The 5'-flanking and 3'-flanking probes are shown, as are the neomycin-resistance gene (*Neo*^r) and loxP sites (triangles). **b-d**, Southern blots of *Bam*HI-digested genomic DNA from wild-type (WT), heterozygous (+/-) and homozygous (-/-) MEFs. **b**, 5' probe; **c**, 3' probe; **d**, *neo* probe. WT and knockout (KO) alleles are indicated by arrowheads. The filled circle indicates a crossreacting band. Molecular weight markers (in kb) are shown. **e**, Northern blot of 20 µg total RNA from MEFs, using an internal fragment of the *LigIV* cDNA (top panel) and reprobing with a phosphoglycerate kinase (PGK) cDNA (bottom panel). A 5-kb

transcript containing 5' and 3' ligase IV sequences interrupted by the *neo*^r gene was also detected (data not shown); however, the *neo*^r sequence contains stop codons in all reading frames in this orientation. 28S and 18S ribosomal RNAs are indicated. **f**, Ligase IV deficiency is embryonic lethal. The numbers of live embryos of each genotype at the indicated stages of development are shown. The numbers of dead embryos are in parentheses. **g**, The genotypes of representative 15-16-day-old embryos are indicated. Analysis of fetal weights of 31 embryos at day 13.5-14.5 (three litters) showed that *LigIV*^{-/-} embryos (seven embryos) were 12% smaller than wild-type and *LigIV*^{+/-} littermates. By 15-16 days, *LigIV*^{-/-} embryos (6 embryos out of 29, from 3 litters) were 25% smaller than their littermates.

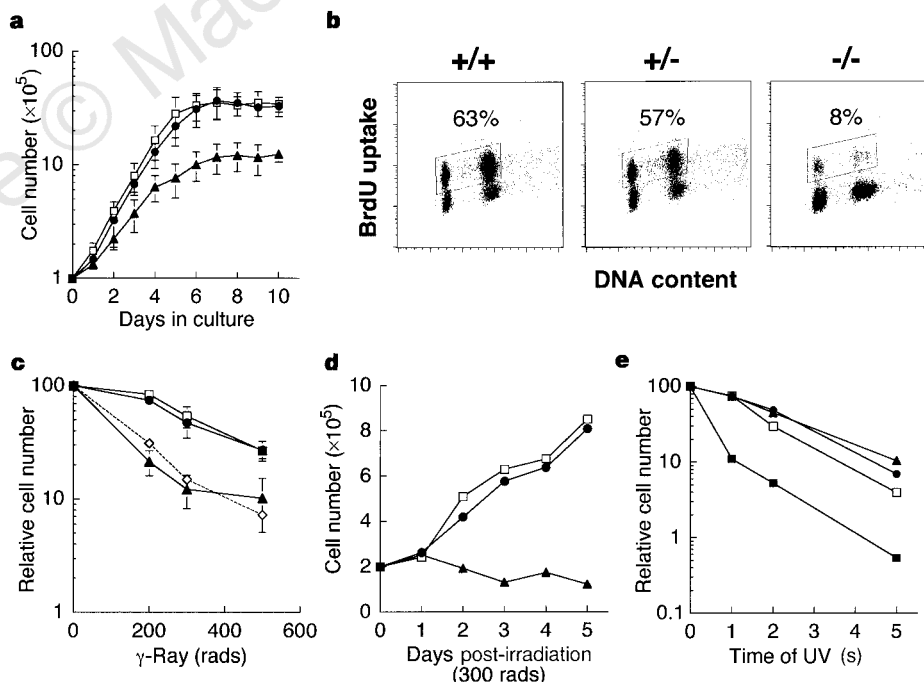


Figure 2 Ligase IV-deficient cells show increased sensitivity to ionizing radiation and a decreased growth potential. **a**, Growth kinetics of primary fibroblasts. Doubling time was 55 h for *LigIV*^{-/-}, 24 h for *LigIV*^{+/-} and 27 h for wild-type MEFs. The average of three assays is shown. WT, filled circles; *LigIV*^{+/-}, open squares; *LigIV*^{-/-}, filled triangles. **b**, Senescence phenotype of *LigIV*^{-/-} MEFs. Day 10 cells from the growth assay were continuously labelled with BrdU. Percentages of actively cycling cells are shown. **c**, Dose response to γ -irradiation. MEFs (passage 3) were exposed to increasing amounts of ionizing radiation, as indicated. The cell count of surviving cells relative to unirradiated cultures of each genotype is plotted as a

function of γ -irradiation. The average of three assays is shown. *DNA-PKcs*^{-/-} MEFs were included in one of the three experiments (open diamonds). **d**, Growth after γ -irradiation. MEFs (passage 3) were treated with 300 rads of γ -irradiation and plated in duplicate. The cell count is plotted as a function of days post-irradiation. The average of two assays is shown. **e**, Dose response to ultraviolet (UV) irradiation. MEFs (passage 3) or human xeroderma pigmentosum cells (filled squares) were irradiated. The cell count of irradiated relative to unirradiated cells of each genotype is plotted as a function of the time exposed to ultraviolet irradiation. One of two similar assays is shown. Symbols in **c-e** are as in **a**.

Table 1 Signal and coding joining in wild-type and *LigIV* mutant MEFs

Cell genotype	Signal joins			Coding joins	
	(A' + C')/A'	Relative level	Fidelity (%)	(A' + C')/A'	Relative level
Experiment 1					
+/+	150/122,900	1.0	100	150/87,500	1.0
+/-	60/129,900	0.38	93	90/99,600	0.59
-/-	0/83,400	<0.01	—	0/106,400	<0.01
Experiment 2					
+/+	1,590/284,000	1.0	100	2,290/331,000	1.0
+/-	2,450/302,000	1.4	100	2,360/337,000	1.0
-/-	0/261,000	<0.0007	—	8/302,000	0.004
Experiment 3					
+/+ (p)	153/51,000	1.0	100	308/71,400	1.0
+/- (p)	137/45,973	1.0	100	254/35,413	1.7
-/- (p)	71/38,493	0.7	92	85/26,540	0.7
Experiment 4					
+/+ (p)	41/19,693	1.0	100	355/61,200	1.0
+/- (p)	278/56,300	2.3	90	1,600/89,500	3.1
-/- (p)	91/62,500	0.7	90	309/18,107	2.9

(A' + C'), number of recombinant colonies resistant to both ampicillin and chloramphenicol; A', number of ampicillin-resistant colonies; (p), pCLig4, a ligase IV cDNA expression plasmid. (A' + C')/A' represents the percentage of V(D)J substrate that recombined.

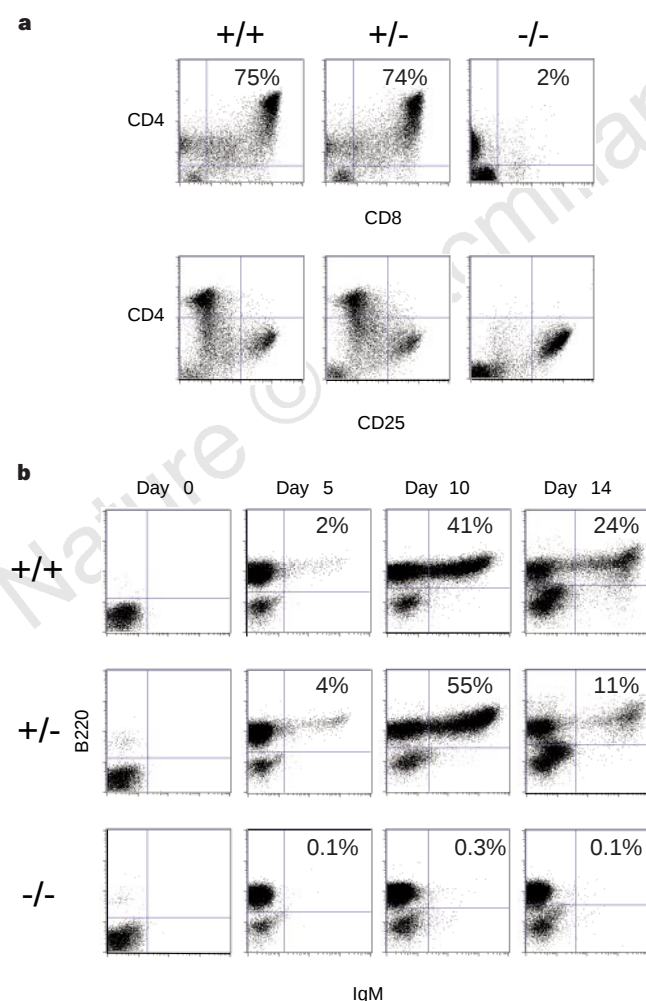


Figure 3 Lymphocyte-development and V(D)J-recombination defects of *LigIV*^{-/-} embryos. **a**, Flow-cytometric analysis of day 16.5–17.5 thymocytes. The percentage of CD4⁺, CD8⁺ or CD4⁺, CD25⁺ cells is shown. These data are representative of 13 wild-type, 29 *LigIV*^{+/-} and 3 *LigIV*^{-/-} embryos. **b**, Flow-cytometric analysis of B cells from cultures of day 15–17.5 fetal livers. These data are representative of 10 wild-type, 21 *LigIV*^{+/-} and 5 *LigIV*^{-/-} embryos. The percentage of B220⁺, IgM⁺ cells is shown.

We used a targeted mutational approach in mice to study mammalian ligase IV functions. We first mapped the ligase IV gene and found that its entire coding sequence is located within a single exon (Fig. 1a). To inactivate this gene in murine embryonic stem cells, we replaced >50% of the ligase IV coding sequences with a neomycin-resistance gene cassette. This targeting event removed nucleotide sequences that encode amino acids 143–608, a region containing critical functional domains including the active-site lysine and conserved catalytic domains that are found in all ATP-dependent DNA ligases (Fig. 1a–d). On the basis of the design of the targeting construct, the ligase IV mutant allele should generate, at most, a short (142-amino-acid) amino-terminal portion of the ligase IV polypeptide and should represent a null allele. Northern blotting analyses with a probe derived from the deleted region of the gene showed that fibroblasts derived from homozygous DNA ligase IV mutant (*LigIV*^{-/-}) embryos (see below) lacked authentic ligase IV transcripts (Fig. 1e).

Mice heterozygous for the germline ligase IV mutation (*LigIV*^{+/-}) were indistinguishable in appearance from wild-type littermates. However, when we intercrossed *LigIV*^{+/-} mice we detected no full-term

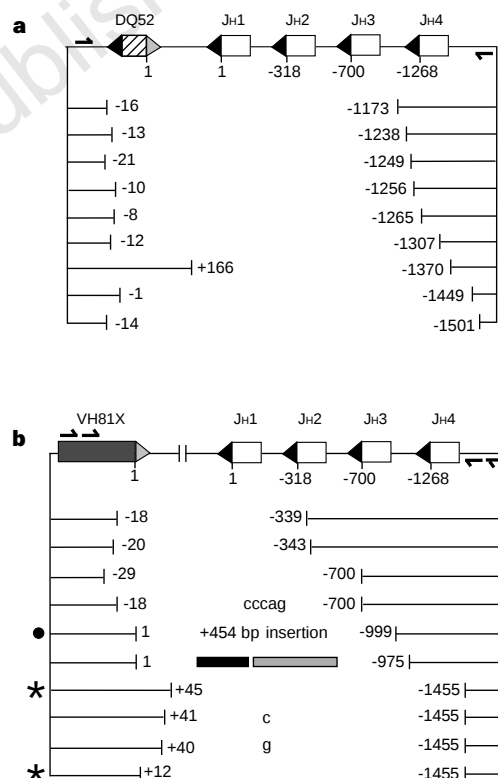


Figure 4 Structure of D to J and V to DJ rearrangements from *LigIV*^{-/-} B cells. Germline loci are shown at the top of **a** and **b**. Position 1 represents the nucleotide directly next to the RSS in each coding segment. **a**, Nine D to J joins were sequenced. Deletions are indicated below the full-length DJ template and are determined relative to position 1 of DQ52 or of JH1. Six of the nine DJ joins were homology-mediated, with 1–5 redundant nucleotides at the junctions. **b**, Ten V to DJ joins were sequenced and are shown below the full-length template. Deletions are determined relative to JH1 and to VH81X or related VH7183 family members, as indicated. N regions, non-templated nucleotides added by terminal deoxynucleotidyl transferase, and insertions are shown in the centre (lower-case letters). The black rectangle in the centre represents an insertion comprising 166 bp of the 5'-flanking region of the D region, FL16.1; the grey rectangle represents 323 bp that are homologous to a transposon-like long-terminal repeat. The nested primer, JH4P, is next to the deletions observed up to -1455. Two of the ten V to DJ joins were homology-mediated and both contained six redundant nucleotides at the junctions. Asterisk indicates a VH7183 family member; circle indicates a VH7183 pseudogene. Primers are indicated as half-arrows.

homozygous *LigIV*^{-/-} mice, indicating that the mutation is embryonic lethal (Fig. 1f). Analyses of embryos from heterozygous mutant crosses at different days of gestation showed that *LigIV*^{-/-} embryos were present in the expected numbers up to 15–16 days of gestation, but were markedly less represented by day 16, at which point we found a significant number of dead *LigIV*^{-/-} embryos (Fig. 1f). Live *LigIV*^{-/-} embryos were, on average, about 25% smaller than littermates at days 15–16; however, the identification of the cause of death will require a more detailed examination as visual inspection of *LigIV*^{-/-} embryos revealed no gross defects (other than a small thymus; see below) and no apparent anaemia (Fig. 1g).

For cellular analyses, we generated fibroblast cell lines (mouse embryonic fibroblasts, MEFs) from 13.5-day-old embryos. Early-passage *LigIV*^{-/-} MEFs had a significantly longer doubling time than *LigIV*^{+/-} or wild-type MEFs (Fig. 2a). In addition, pulse labelling of these MEFs with 5-bromodeoxyuridine (BrdU) showed that 23–34% fewer cells in the *LigIV*^{-/-} cultures were in the S phase of the cell cycle as compared with wild-type or *LigIV*^{+/-} MEFs (data not shown). Continuous BrdU labelling of day 10 cells from the first growth assay (Fig. 2a) showed that markedly fewer *LigIV*^{-/-} cells were actively cycling compared with *LigIV*^{+/-} or wild-type cells (Fig. 2b). Also, when replated at low density, these day 10 *LigIV*^{-/-} MEFs showed a pronounced inability to proliferate in comparison with *LigIV*^{+/-} and wild-type MEFs (data not shown). These results indicate that *LigIV*^{-/-} MEFs, like *Ku70*^{-/-} and *Ku80*^{-/-} MEFs^{4,6}, lose proliferative capacity and become senescent prematurely.

To assess the role of DNA ligase IV in DNA-damage repair, we exposed MEFs to increasing amounts of ionizing radiation and found that *LigIV*^{-/-} cells exhibited marked sensitivity (Fig. 2c, d). *LigIV*^{-/-} cell growth, in contrast to that of wild-type or *LigIV*^{+/-} cells, was severely blocked when the cells were treated with an intermediate dose of ionizing radiation (Fig. 2d). In fact, *LigIV*^{-/-} MEFs appeared to be at least as sensitive to ionizing radiation as DNA-PKcs-deficient MEFs (Fig. 2c). To determine the specificity of the *LigIV*^{-/-} DNA-repair defect, we tested the sensitivity of *LigIV*^{-/-}, *LigIV*^{+/-} and wild-type MEFs to ultraviolet light and found no differences; we used a human xeroderma pigmentosum skin fibroblast cell line (XP-25) as an ultraviolet-hypersensitive control (Fig. 2e). We conclude that ligase IV is a major component of the machinery used to repair ionizing-radiation-induced DNA damage in mammalian cells, but has no major role in the repair of ultraviolet-induced DNA lesions.

To determine whether or not ligase IV is involved in *V(D)J* recombination, we used a transient transfection assay^{12,22} in which MEFs were transfected with RAG1 and RAG2 expression vectors and with transient *V(D)J* recombination substrates, which allow detection of either coding or RSS joins. For *LigIV*^{-/-} MEFs, we observed no recovery of RSS joins in any of the six assays performed and a low level of potential coding joins in only one assay, whereas *LigIV*^{+/-} MEFs showed levels of RSS and coding joins that were similar to wild-type levels (Table 1). Furthermore, analyses of the few potential coding joins recovered from *LigIV*^{-/-} cells revealed rearrangements that did not correspond to RAG-specific RSS cleavages (data not shown). In confirmation of the idea that the defect in *V(D)J* recombination resulted directly from the lack of an active DNA ligase IV protein, we found that a full-length murine DNA ligase IV DNA expression construct complemented both the RSS- and the coding-joining deficiencies of the *LigIV*^{-/-} cells, resulting in levels similar to those of wild-type and *LigIV*^{+/-} cells (Table 1).

We assessed the role of DNA ligase IV in *V(D)J* recombination *in vivo* by studying lymphocyte development in *LigIV*^{-/-} embryos. Comparison of thymocyte populations of *LigIV*^{-/-}, *LigIV*^{+/-} and wild-type embryos at embryonic day 16.5–17.5 showed that *LigIV*^{-/-} thymocyte development was arrested at the CD4⁻, CD8⁻, CD25⁺ stage (Fig. 3a), consistent with a defect in productive rearrangement of T-cell antigen-receptor- β genes. To further examine lymphocyte

development, we cultured fetal liver cells from day 15–17.5 embryos under conditions that allow differentiation of progenitor B cells. By day 10, the wild-type and *LigIV*^{-/-} cultures had a large percentage of IgM⁺, B220⁺ B cells, whereas the *LigIV*^{-/-} cultures had very few, indicating a clear defect in B-cell development (Fig. 3b). We isolated potential *D* to *J_H* and *V_H* to *D_HJ* joins in DNA from day 14 *LigIV*^{-/-} B-cell cultures by polymerase chain reaction. Nucleotide sequencing of these rearrangements revealed attempted *D_J* and *V(D)J* joins that were generally grossly aberrant, with large deletions extending from one or both sides of the recombining segments (Fig. 4a, b), similar to the situation in Ku-deficient^{6,12,23} and DNA-PK-deficient^{12,24,25} cells. However, the finding that some recombination points were immediately adjacent to the RSS (Fig. 4a, b) indicates that cutting by RAGs can occur normally in *LigIV*^{-/-} progenitor lymphocytes and that the defect is in the subsequent joining phase of the reaction.

We conclude that DNA ligase IV provides the major ligation activity for the joining of RSS and coding ends during physiological *V(D)J* recombination and that this enzyme is also required for the repair of DNA damage induced by ionizing radiation. Our finding that DNA ligase IV is absolutely required in *V(D)J* recombination, both *in vivo* and in cell lines, does not support the idea that DNA ligase I provides the major ligation activity for *V(D)J* recombination²¹, as ligase IV-deficient cells must have relatively normal ligase I function to allow DNA replication. However, we cannot rule out the possibility that ligase I could have some role in this process. For example, end-joining could conceivably use sequential activities in which ligase IV would seal one strand of the break and a second ligase would complete the reaction in a replication/repair process.

With respect to effects on *V(D)J* recombination, cell growth and sensitivity to ionizing radiation, the ligase IV-deficient phenotype appears to closely resemble that of Ku80- and Ku70-deficient mice^{3–6}. Therefore, most known defects of Ku-deficient mice could be explained by the presence of an impaired end-joining process that requires ligase IV in addition to the Ku proteins. However, the phenotype of ligase IV-deficient mice is more severe than that of Ku-deficient animals, as the latter are viable, albeit smaller than normal. Therefore, ligase IV may have additional functions, perhaps in association with other proteins, or Ku deficiency may produce a more 'leaky' end-joining phenotype.

Note added in proof: An embryonic-lethal phenotype has also been observed in an independent ligase IV knockout (T. Lindahl, personal communication). □

Methods

Generation of mice deficient in DNA ligase IV. We screened a 129/sv mouse genomic library (Stratagene) and a murine testis complementary DNA library (Clontech), and then identified one exon that includes 29 base pairs (bp) of the 5'-untranslated region and the entire coding region of the DNA ligase IV gene. To generate the targeting construct, we inserted a 5.6-kilobase (kb) *HindIII*–*HindIII* genomic fragment containing 5' flanking sequence and 456 bp of the ligase IV coding sequence into an *XhoI* site of the pLNTk vector (pSall–*loxP*–*neo'*–*loxP*–*XhoI*–*tk* cassette vector)²⁶. A 2.4-kb *HindIII*–*HindIII* genomic fragment containing 3'-flanking sequence and 910 bp of the exon was inserted into the *Sall* site of this vector. Embryonic stem cells (TC1, derived from 129/sv mice, provided by P. Leder) were transfected with the targeting vector as described²⁷. Homologous recombinants were identified by Southern blot analysis using three probes. Nine independently targeted embryonic stem cell clones were injected into C57B1/6 blastocysts to generate chimaeric mice and four embryonic stem cells transmitted the mutation into the mouse germ line, resulting in a 129/sv × C57B1/6 background²⁸. *LigIV* heterozygous mice were intercrossed and the embryos at 13.5 days after plug formation were isolated to make mouse embryonic fibroblasts²⁸.

Transient assays. The transient *V(D)J* recombination assay has been described^{12,22}. After amplification of the signal joins by the polymerase chain reaction (PCR), the fidelity of joining was determined by digestion with *Apal*LI

which cleaves at the site created by a precise junction. The fidelity of 10–15 clones was analysed for each percentage shown in Table 1. The complementing plasmid, pCLig4, contained the full-length murine *LigIV* cDNA subcloned into the pCDNA3 vector. Representative assays are shown in Table 1.

Irradiation-sensitivity assays. Passage 3 MEFs (1×10^5) were treated with 0, 200, 300 or 500 rads of γ -irradiation from a ^{137}Cr source, and plated onto gelatinized dishes of 10 cm in diameter. After 1–5 days, the cells were trypsinized, stained with trypan blue and counted. MEFs (passage 3) and a human xeroderma pigmentosum skin fibroblast cell line (XP-25) were plated at 1×10^5 cells onto gelatinized 10-cm dishes. After the cells attached, the media was replaced with 3 ml PBS and the cells were irradiated with a 30-watt, shortwave ultraviolet light source (254 nm) from a distance of 1 m for the indicated times. After 7 days, the cells were trypsinized, stained with trypan blue and counted.

Growth assays. Passage 3 MEFs (1×10^5) were plated in duplicate onto gelatinized 10-cm dishes and the medium was changed every other day. The cells were trypsinized, stained with trypan blue and counted every 24 h. The doubling times were determined on the basis of the linear portions of the curves. MEFs were plated into 6-well dishes at 2×10^5 to 5×10^5 cells per well. For pulse labelling, BrdU ($10 \mu\text{M}$, Sigma) was added directly to the media for 1 or 2 h after the MEFs (passage 3) had attached. For continuous labelling, the cells were plated with BrdU ($100 \mu\text{M}$) and labelled for 48 h. The cells were collected and then stained with fluorescein isothiocyanate (FITC)-conjugated anti-BrdU antibodies and propidium iodide according to the manufacturer's protocol (Becton Dickinson).

Lymphocyte development. Cells from day 16.5–17.5 fetal thymi were stained with anti-CD25 (clone 7D4), anti-CD8 (clone 53-6.7) and anti-CD4 (clone GK1.5) antibodies (Pharmingen) conjugated with FITC, cytochrome C or phycoerythrin. 1×10^6 cells from day 15–17.5 fetal livers were plated on T220 stromal cells, an interleukin-7-secreting cell line, in 6-well plates, with 2–3 wells per time point. The cells were stained with $1 \mu\text{g ml}^{-1}$ anti-IgM-phycoerythrin (goat anti-mouse polyclonal) and anti-B220-FITC (clone RA3-6B2) antibodies (Pharmingen) and propidium iodide (Sigma) on days 0, 5, 10 and 14. Data were collected on a FACSCalibur flow cytometer (Becton Dickinson) using Cell Quest software (Becton Dickinson) and analysed using FlowJo software (Tree Star).

PCR analysis of D to J and V to DJ rearrangements. We isolated DNA from fetal liver cells after 14 days. IgH *DJ* rearrangements were PCR-amplified using primers DQ52 (5'-CCACAGGCTCGAGAACTTTAGCG-3') and JH4 (5'-TCCTCAAA TGAGCCTCAAAAGTCC-3'). IgH *V-DJ* rearrangements were PCR-amplified using primers FW.1 (5'-GTGGAGTCTGGGGGAGGCTTA-3') and JH4 and nested primers CDR2 (5'-AATAGTGATGGTGGTAGCACC-3') and JH4P (5'-ATAA TCTGTCCTAAAGGCTCTG-3'). The amplified products were cloned into the pCR2.1-TOPO (Invitrogen), pGEM (Promega) and pSKBluescript (Stratagene) vectors, and individual clones were isolated for sequencing.

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Correspondence and requests for materials should be addressed to F.W.A. (e-mail: alt@rascal.med.harvard.edu).

The murine gene *p27^{Kip1}* is haplo-insufficient for tumour suppression

Matthew L. Fero*, Erin Randel*, Kay E. Gurley*, James M. Roberts*† & Christopher J. Kemp*

* Fred Hutchinson Cancer Research Center, 1100 Fairview Avenue North, Seattle, Washington 98109, USA

† Howard Hughes Medical Institute, Chevy Chase, Maryland 20815, USA

p27^{Kip1} is a candidate human tumour-suppressor protein, because it is able to inhibit cyclin-dependent kinases and block cell proliferation^{1–5}. Abnormally low levels of the p27 protein are frequently found in human carcinomas, and these low levels correlate directly with both histological aggressiveness and patient mortality^{6–10}. However, it has not been possible to establish a causal link between p27 and tumour suppression, because only rare instances of homozygous inactivating mutations of the p27 gene have been found in human tumours^{11–14}. Thus, *p27^{Kip1}* does not fulfil Knudson's 'two-mutation' criterion for a tumour-suppressor gene¹⁵. Here we show that both p27 nullizygous and p27 heterozygous mice are predisposed to tumours in multiple tissues when challenged with γ -irradiation or a chemical carcinogen. Therefore p27 is a multiple-tissue tumour suppressor in mice. Molecular analyses of tumours in p27 heterozygous mice show that the remaining wild-type allele is neither mutated nor silenced. Hence, p27 is haplo-insufficient for tumour suppression. The assumption that null mutations in tumour-suppressor genes are recessive excludes those genes that exhibit haplo-insufficiency.

Tumour-suppressor proteins affect several cellular pathways, such as those controlling proliferation, apoptosis, differentiation and genomic integrity. Consequently, the identification of a tumour-suppressor gene is usually established genetically rather than by any particular functional criterion. The common operational definition of a tumour-suppressor gene requires the demonstration of mutations of both copies of a candidate gene in tumours¹⁵. Genetic and physical mapping of bi-allelic tumour-

specific mutations allows localization of tumour-suppressor genes, and provides evidence for a causal link between mutations in those genes and tumorigenesis. This approach has successfully demonstrated the tumour-suppressor function of the retinoblastoma protein (Rb), p53, INK4a, and others^{16–18}. However, we show here that this definition is too restrictive because it excludes tumour suppressors such as p27^{Kip1} that exhibit haplo-insufficiency.

We studied the tumour-suppressor function of p27^{Kip1} by examining the susceptibility of p27-deficient mice to tumorigenesis induced by two different DNA-damaging agents, γ -irradiation and the chemical carcinogen *N*-ethyl-*N*-nitrosourea (ENU). p27^{-/-} mice showed decreased tumour-free survival following γ -irradiation compared with p27^{+/+} controls (Fig. 1a). p27^{+/-} mice showed an intermediate sensitivity (see below). Thymic lymphomas were the most frequent tumours in wild-type mice (incidence 0.07), but their frequency was not significantly increased in p27^{-/-} animals (incidence 0.17). The increased mortality of p27-null mice was mainly due to the development of pituitary tumours and intestinal adenomas (Fig. 2). A separate group of mice was injected with a single dose of ENU, and again p27-null animals showed increased tumour-related mortality (Fig. 1b). In p27^{+/+} mice, lymphoma incidence (0.26) and liver adenomas were the most frequent causes of tumour morbidity. The frequency of these tumour types was not increased in p27^{-/-} animals (lymphoma incidence 0.23). Instead, the increased tumour-related mortality in p27^{-/-} mice following ENU treatment was mainly attributable to increased numbers of adenomas of the intestine and pituitary (Fig. 2). Not all tumours caused morbidity: some were identified concurrently at necropsy or at the termination of the experiments (see Methods). Concurrent tumours had less time to develop in p27-deficient animals compared with controls because of their shorter lifespan. Hence, the experimental design underestimated the contribution of p27 deficiency to tumorigenesis.

Lesions of female reproductive organs also occurred more frequently in p27-deficient mice (Fig. 2), and were usually associated with histological evidence of neoplasia. These lesions included granulosa cell tumours of the ovary, endometrial adenocarcinomas, endometrial polyps, angiosarcomas, and fibromas. Adrenal tumours, both medullary and cortical, occurred at higher frequency in p27-deficient animals (one p27^{+/+}, four p27^{+/-}, and four p27^{-/-} mice in both experiments combined).

In p27^{-/-} mice, the incidence of adenomas in both the small and the large intestine was increased (Fig. 2), as was the number of malignant tumours; 7 out of 46 (15%) of the p27-null animals but just 1 out of 63 (1.6%) of p27^{+/+} controls ($P < 0.05$, chi-squared test) developed locally invasive intestinal tumours, defined as invasion of neoplastic epithelia through the full thickness of the muscularis, indicating progression to adenocarcinoma. A smaller

number of intestinal stromal sarcomas also occurred late in the ENU-exposed animals, but these were independent of p27 genotype.

The number of lung adenomas was increased in p27^{-/-} animals following both irradiation and ENU treatment (Fig. 2) and ranged in size from 1 to 5 mm. In three p27-null animals but in none of the wild-type controls the lung tumours completely consolidated one or more lung lobes and exhibited dysplasia typical of adenocarcinomas. Pituitary tumours in p27-deficient mice were intermediate melanotroph adenomas, similar to those seen in untreated p27 knockouts⁵.

p27^{+/-} mice were also significantly more susceptible than wild-type mice to γ -irradiation and ENU-induced tumorigenesis of the lung, intestine, and pituitary, but at intermediate rates (Fig. 2). The tumours were histologically similar to those in p27^{-/-} animals. Increased tumorigenesis is often observed in mice heterozygous for a germline deletion of a tumour-suppressor gene, and in these cases, in keeping with the two-mutation model, the remaining wild-type allele is inactivated^{19–22}. In contrast, no deletions or rearrangements of the wild-type p27 allele were found in 33 tumours from p27 heterozygotes, including 15 samples of lung, intestine and pituitary obtained after both X-ray and ENU treatments (Fig. 3a, and results not shown). Of 13 tumours assessed further by DNA sequencing, no point mutations were present in the p27 gene's coding region. Moreover, the p27 protein was present at significant levels in 23 p27-heterozygote tumours analysed by immunoblot (Fig. 3b–d, and results not shown). Five lung and four pituitary tumours that had been analysed by Southern blotting and DNA sequencing were also analysed by quantitative immunoblotting, which showed that the amount of p27 protein closely approximated that found in normal heterozygous tissue, which is 50% of the level in wild-type tissue (Fig. 3c, d). Immunostaining for p27 protein

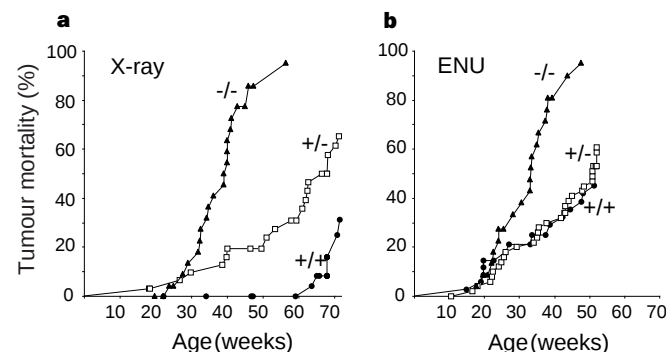


Figure 1 Tumour-induced mortality according to p27 genotype. **a**, γ -irradiation-induced tumour mortality (all tumour types) is increased in both p27^{-/-} and p27^{+/-} mice. **b**, ENU-induced tumour mortality is increased in p27^{-/-} mice. Non-tumour-related mortality was censored at the time of death (see Methods).

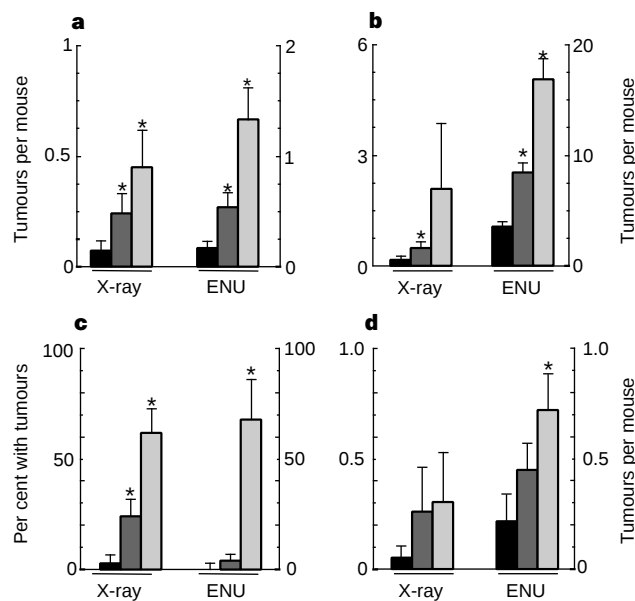


Figure 2 Cumulative occurrence of specific tumour types. The mean number of tumours ($\pm$ s.e.m.) per mouse (tumour multiplicity) following treatment with either γ -irradiation or ENU is plotted for p27^{+/+} (black bars), p27^{+/-} (grey bars), and p27^{-/-} (striped bars) mice. **a**, Intestinal adenomas; **b**, lung adenomas; and **d**, tumours of the female reproductive tract. (Asterisks indicate $P < 0.05$ compared with wild-type by Wilcoxon rank sum test.) **c**, The occurrence of pituitary tumours is plotted as percentage incidence. (Asterisk indicates $P < 0.05$ compared with wild-type controls by chi-squared test.)

showed that p27 protein was expressed in the nuclei of the tumour cells (Fig. 3e). Intestinal tumours contained amounts of p27 protein that were greater than those in normal p27^{+/+} intestine (Fig. 3b), indicating that expression of p27 in the intestine as a whole is not representative of its expression in the cell of origin of these tumours. However, the amount of p27 protein in these tumours was half of that in an intestinal tumour from a wild-type mouse, indicating that expression from the intact allele in the p27^{+/-} tumours was not downregulated (results not shown).

p27 was also haplo-insufficient for suppressing spontaneous tumorigenesis. p27^{+/-} mice spontaneously developed melanotroph tumours in the pituitary with a penetrance of 32% ($n = 38$) and a median latency of 19 months, whereas wild-type littermates developed no pituitary tumours ($n = 42$). We found no deletions or silencing of the wild-type p27 allele when we analysed four of these tumours from heterozygous mice (Fig. 3f, g). We sequenced the coding region of the p27 gene from one of these tumours, and found no mutations. Rb^{+/-} mice also spontaneously develop melanotroph tumours in the pituitary; however, in those tumours the remaining wild-type Rb allele was always lost^{21,22}. p27 contrasts with Rb in this regard; our results show that p27 does not conform to the paradigm of two-hit inactivation in tumours.

Our results suggest that decreased p27 protein expression and human tumour progression are causally related. The sensitivity to p27 dosage suggests that decreased p27 function may be a common event in sporadic human tumours. A twofold drop in p27 expression could occur either by heterozygous gene mutation or by changes in extragenic pathways that modulate p27 protein levels. Evidence from studies of humans supports both of these possibilities. More than 50% of primary breast cancers expressed low amounts of the p27 protein, and similar results have been obtained for colon, gastric and prostate tumours⁶⁻¹⁰. This is likely to be

the result of defects in pathways that regulate p27⁷. Hemizygous deletions at chromosome 12p13, which include the p27^{Kip1} gene, have also been seen in primary human tumours^{11,14,23-25}. Inactivation of both p27 alleles would be relatively rare, and may not provide sufficient advantage over the heterozygous state to be strongly selected in tumours.

The molecular basis for p27 haplo-insufficiency is not yet understood. Decreased amounts of p27 may alter the stoichiometric relationship between itself and cyclin-dependent kinases (CDKs), and this could underlie the proliferative abnormalities in p27-deficient cells. This suggests that the haplo-insufficient phenotypes of p27 could be enhanced by mutations in genes that modulate cyclin expression and CDK activity. The importance of haplo-insufficiency in tumorigenesis is also shown by the tumour predisposition of mice heterozygous for a transforming growth factor- β (TGF- β) deletion²⁶. However, because TGF- β is a secreted protein, its tumour-inhibitory effect depends on its expression in the whole animal, not by the tumour cell (that is, it does not produce a cell-autonomous phenotype). Therefore, the significance of this example lies in understanding tumour-prone phenotypes in the animal, as opposed to the genetic changes that occur within the tumour cell.

Our results establish a new class of tumour-suppressor genes that are haplo-insufficient, p27^{Kip1} being one example. Inheritance of just one p27 allele resulted in a tumour-prone phenotype, and tumour cells arising in p27^{+/-} mice did not show mutation or silencing of expression of the intact p27 gene. Genes exhibiting haplo-insufficiency may be frequent targets during the evolution of tumour cells, because inactivation of only one allele or a moderate decrease in protein expression is sufficient to predispose to tumorigenesis. Deletion of one p53 allele has been shown to compromise its biological functions *in vivo*, indicating that haplo-insufficient phenotypes may be widespread²⁷⁻²⁹.

A potentially important strategy for cancer therapeutics will be to restore expression of tumour-suppressor proteins. Haplo-insufficient tumour suppressors such as p27 may be useful in achieving this goal, because they retain a functional allele in tumours. □

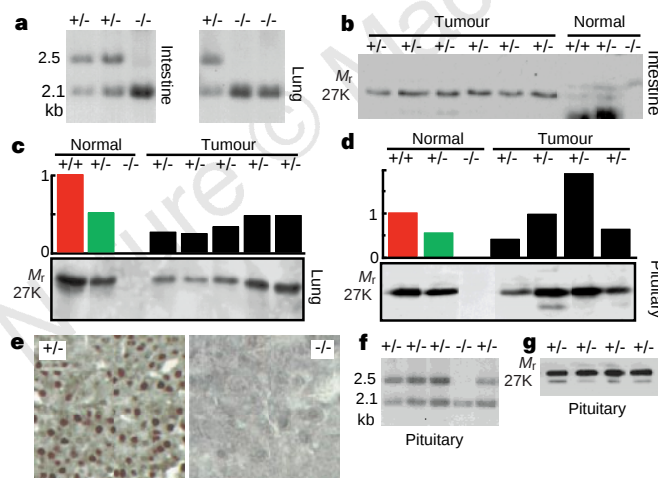


Figure 3 Analysis of tumours from p27^{+/-} mice. **a**, Representative Southern blots of ENU-induced intestinal and lung adenomas are shown with the genotype of the mouse listed above. The wild-type allele corresponds to the 2.5-kb band, and the knockout allele to the 2.1-kb band. No evidence of allelic loss is seen in p27^{+/-} tumours. **b-d**, Immunoblots of p27 protein expression in normal intestine, lung and pituitary, compared with p27 expression in tumours arising in these tissues following ENU treatment (intestine and lung) and γ -irradiation (pituitary). In **c**, **d**, the expression of p27 was quantified by densitometry followed by normalization to the amount of tubulin present in each sample (see Methods). Plotted are levels of protein in tumours relative to levels in normal, wild-type tissue. **M_r**, apparent relative molecular mass. **e**, Immunostaining of p27 protein in a p27^{+/-} pituitary tumour induced by γ -irradiation (compared with p27^{-/-} control). The p27 protein is localized to the nuclei of the tumour cells. **f**, Southern blot of spontaneous pituitary adenomas, with the corresponding genotype of the mouse listed above. **g**, Immunoblot of p27 protein expression in the same p27^{+/-} tumours as in **f**. The amount of p27 protein is similar to that present in normal p27^{+/+} pituitary tissue.

Methods

Mice. All of the animals studied were littermates produced from heterozygous crosses. An untreated cohort including each genotype was observed for 2 years and comprised 25 F₁ and F₂ generation C57B6/J $\times$ 129/Sv hybrids, plus 15 more inbred animals (congenic to the founding 129/Sv embryonic cell line). For the X-ray study, C57BL/6J $\times$ 129/Sv F₂ generation hybrids were used as breeders. Mice for the experiment with ENU were produced by first backcrossing the original p27 chimera to C57BL/6J mice for six generations. These were bred to p27^{+/-} 129/Sv inbred mice to produce F₁ hybrids. All mice were genotyped by the polymerase chain reaction (PCR) as described⁹.

Treatment. The 94 mice included in the X-ray experiment were treated with 4 Gy whole-body γ -irradiation (with a ¹³⁷C source, 330 cGy per second) at the age of 14 $\pm$ 2 days, and were weaned at 3–4 weeks. Twenty-nine +/+, thirty-two +/-, and twenty-five -/- mice were included in the experiment. There was no increase in early mortality associated with the radiation treatment. In a separate experiment, 109 littermates (34 p27^{+/+}, 51 p27^{+/-} and 24 p27^{-/-}) were injected intraperitoneally with ENU (0.5 μ mol per g mouse) at the age of 15 $\pm$ 2 days, and were weaned at 3–4 weeks.

Histological analysis. Complete necropsies were done at the first sign of morbidity, or when visible tumours were >1 cm in size. All remaining animals were necropsied at the termination of the experiment (16 months following irradiation, or 12 months following ENU treatment). Mortality was scored as tumour-related if the tumour was large (≥ 1 g) or if secondary pathophysiology was present. Non-tumour-related morbidity occurred in ~10% of animals, independent of genotype, and consisted primarily of ocular and skin lesions. Tumours greater than 1 mm in size were quantified. Haematoxylin-and-eosin-stained sections were separately assessed by M.L.F. and C.J.K. and discrepancies of interpretation were resolved through an independent pathologist's review. Separate sections were immunostained after antigen retrieval over a steaming

citrate (0.01 M, pH6) bath, followed anti-p27 monoclonal antibody (1:50; NeoMarkers), biotinylated anti-IgG1 secondary antibody (1:200; Southern Biotech), StreptABC-HRP (DAKO), and colour development using DAB/NiCl.

Biochemical analysis. Tumours were selected for biochemical analysis if they were of sufficient size and histological homogeneity (>95% homogeneity for lung and pituitary adenomas). Intestinal tumours selected for analysis consisted of pedunculated polyps which could be easily excised away from their stalk of normal tissue. Protein extracts were prepared from frozen specimens by homogenization in buffer containing protease inhibitors. Protein content was standardized between samples by Bradford assay (BioRad) and by staining the gel with Coomassie blue. p27 protein expression was determined through immunoblot using a rabbit polyclonal anti-p27 antisera as described⁸. Autoradiographs of p27 immunoblots were scanned and quantified (NIH Image). A p27 standard curve generated by serial dilution was used to ensure that all samples were within the linear range of the assay. p27 protein levels were then normalized to the amount of tubulin protein present on the same filters (anti-tubulin monoclonal antibody; BAbCo). Tumours were genotyped by Southern blot with the 3' p27 genomic probe, using *Xba*I and *Xho*I restriction digests, as described⁹. Tumours were also sequenced using PCR to amplify and the two coding exons of p27 from 20 ng tumour DNA with nested primers. Fifteen cycles were performed using primers K15 (5'-TTCGAAGAGGG-TTTTGCCTCCAT-3') and K26 (5'-CCAGATGGGGTGTGAGTTTGTGT-3'), followed by 25 cycles with K16 (5'-GAGAGGCGAGGCGGTGGTCC-3') plus K25 (5'-GCTGTTTACGTCTGGCGTCA-3'). Dye-terminator cycle sequencing (Perkin Elmer) was used with primers for both coding exons. Sequences were compared with the published complementary DNA sequence for mouse p27 and with sequence data we obtained from wild-type 129/Sv and C57BL/6J mice³⁰. C57BL/6J wild-type mice had the following p27 polymorphism: 66T → G, D22E; 422A → C, Q141P.

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Correspondence and requests for materials should be addressed to C.J.K. (e-mail: cj Kemp@fhcrc.org).

The protein kinase Pak3 positively regulates Raf-1 activity through phosphorylation of serine 338

Alastair J. King^{*,†}, Huaiyu Sun^{‡,†}, Bruce Diaz^{*,†}, Darlene Barnard^{*,†}, Wenyan Miao^{‡,†}, Shubha Bagrodia[§] & Mark S. Marshall^{*,†,‡}

^{*}Department of Medicine, Division of Hematology/Oncology, [‡]Department of Biochemistry & Molecular Biology, Indiana University School of Medicine and [†]The Walther Oncology Center, Indianapolis, Indiana 46202, USA
[§]Department of Pharmacology, Section of Molecular & Cell Biology, Cornell University, Ithaca, New York 14853, USA

The pathway involving the signalling protein p21^{Ras} propagates a range of extracellular signals from receptors on the cell membrane to the cytoplasm and nucleus¹. The Ras proteins regulate many effectors, including members of the Raf family of protein kinases. Ras-dependent activation of Raf-1 at the plasma membrane involves phosphorylation events, protein–protein interactions and structural changes^{2–8}. Phosphorylation of serine residues 338 or 339 in the catalytic domain of Raf-1 regulates its activation in response to Ras, Src and epidermal growth factor^{9,10}. Here we show that the p21-activated protein kinase Pak3 phosphorylates Raf-1 on serine 338 *in vitro* and *in vivo*. The p21-activated protein kinases are regulated by the Rho-family GTPases Rac and Cdc42 (ref. 11). Our results indicate that signal transduction through Raf-1 depends on both Ras and the activation of the Pak pathway. As guanine-nucleotide-exchange activity on Rac can be stimulated by a Ras-dependent phosphatidylinositol-3-OH kinase^{12,13}, a mechanism could exist through which one Ras effector pathway can be influenced by another.

A study of rat organs indicated that the spleen was the best source of a protein kinase activity capable of phosphorylation of serine in a peptide corresponding to Raf-1 residues 331–345. We purified this kinase activity from the cytosolic fraction of rat spleen homogenates by sequential column chromatography (Fig. 1). We used a Mono-Q matrix to resolve this activity into three major peaks of Raf-1-peptide kinase activity, only one of which was shown by two-dimensional phosphopeptide mapping to be specific for the Raf-1 S338/339 site in a recombinant Raf-1 catalytic fragment (Raf-CR3) (data not shown). The kinase was further purified by isocratic elution from Mono-Q and finally by passage over a Mono-P column. Kinase activity eluted with an isoelectric point of 5.95 and consisted of a near-homogenous protein of relative molecular mass 37,000 (*M*_r 37K), termed p37, as assessed by silver-stained SDS–polyacrylamide gel electrophoresis (PAGE) (Fig. 1a, sample P). An extremely high specific activity (19,500 pmol min^{−1} mg^{−1}) was achieved, representing a 6,132-fold purification.

We examined the purified kinase in an in-gel kinase assay, using myelin basic protein as the substrate; this assay showed two reproducible bands of kinase activity (Fig. 1b). One band was a 37K band, confirming the identity of purified p37 as a protein kinase. The 40K activity, also faintly visible in sample P of Fig. 1a, was later shown to be immunologically identical to p37 (see below). We confirmed the specificity of the purified kinase for Raf-1 S338/339 by using as substrates Raf-CR3 and Raf-CR3^{D338E339}, the latter lacking a phosphorylatable 338/339 site (Fig. 1c). The purified kinase phosphorylated wild-type Raf-CR3 but failed to significantly phosphorylate the Raf-CR3^{D338E339} mutant; phosphoamino-acid analysis revealed only phosphoserine (Fig. 1d). Two-dimensional phosphopeptide mapping of Raf-CR3 phosphorylated by the purified kinase identified the same tryptic phosphopeptides that were shown previously to contain S338/339 (Fig. 1e)⁹. These peptides contained >95% of the ³²P incorporated within phosphorylated Raf-CR3. Phosphopeptide mapping of Raf-CR3^{D338E339} from parallel incubations revealed only the weakly phosphorylated background peptides that were also found in phosphorylated wild-

type Raf-CR3 (data not shown). We also confirmed that the S338/339 site was phosphorylated by specific immunoprecipitation of the S338/339 tryptic phosphopeptides generated from phosphorylated Raf-CR3 using a polyclonal antibody raised against residues 337–354 of Raf-1 (ref. 9). Immune, but not preimmune, serum precipitated these phosphopeptides, further identifying S338/339 as the site of specific phosphorylation within Raf-CR3 (Fig. 1f). The occurrence of multiple phosphopeptides correlating with S338/339 phosphorylation has been explained⁹.

We then identified the purified 37K protein by peptide microsequencing. p37 obtained from a large-scale preparation was eluted from a preparative gel and fragmented by digestion with trypsin. The peptides were purified by high-pressure liquid chromatography (HPLC) and four of the HPLC-purified polypeptides were sequenced by mass-spectrometric microsequence analysis. The amino-acid sequences of these polypeptides were compared with the SWISS-PROT protein-sequence database entries and gave a complete match with regions of the catalytic domain of the p21-activated protein kinase isoform Pak3 (Fig. 2). Hence, from the sequences obtained we deduce that the purified 37K Raf-1 S338/339 protein kinase is a proteolytically cleaved catalytic-domain fragment of Pak3. Immunoblot analysis of the Mono-Q extract with a Pak3 carboxy-terminal-specific antibody confirmed that both the 37K and the 40K kinase activities represent proteolytic-degradation fragments of full-length Pak3 (data not shown).

After identifying p37 as a fragment of Pak3, we tested the ability of full-length Pak3, immunoprecipitated from rat spleen lysates, to phosphorylate Raf-1 S338/339. Immunoprecipitates were initially assessed for kinase activity towards the Raf-1(331–345) peptide (Fig. 3a). Immunoprecipitated Pak3 was capable of incorporating fourfold more phosphate into wild-type Raf-CR3 than into the non-phosphorylatable Raf-CR3^{D338E339} or Raf-CR3^{S338A339} proteins (Fig. 3b). We obtained similar results when using recombinant Pak3, prepared from Sf9 cells. The Raf-1 S338 kinase activity of recombinant Pak3 was stimulated up to sixfold by incubation with GST–Rac-GTPγS or GST–Cdc42-GTPγS (Fig. 3c, d). Furthermore, the p21-GTP-stimulated activity of Pak3 was directed specifically towards S338 (Fig. 3d). These results confirm the identity of the purified Raf-1 S338/339 kinase as a catalytic fragment of Pak3, rather than an undetected minor protein kinase species. Immunodepletion of the purified rat spleen kinase with Pak3-specific

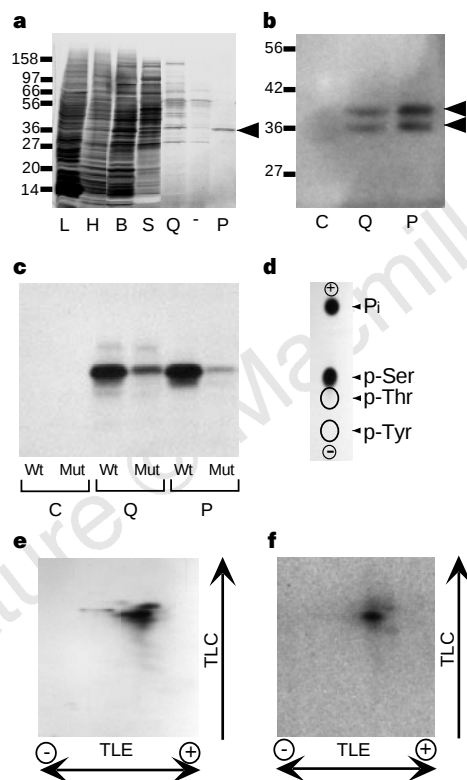


Figure 1 Raf-1 S338/339 kinase was purified from rat spleen by sequential chromatography and identified as a specific Raf-1 S338/339 kinase. **a**, Kinase purification as resolved by SDS-PAGE and stained with silver. Crude spleen lysate (L), heparin-Agarose (H), blue-Sepharose (B), Sephacryl Hi-Prep S-200 (S), Mono-Q (Q) and Mono-P (P) eluates are shown. Relative molecular masses are in K. – denotes lane is not relevant to the figure. Arrowhead indicates p37. **b**, In-gel kinase assay with myelin basic protein substrate. Control (C), Mono-Q (Q) and Mono-P (P) extracts are shown. Arrowheads indicate the two bands of kinase activity. **c**, Confirmation of S338/339 phosphorylation, using Raf-CR3 (Wt) and Raf-CR3^{D338E339}, a non-phosphorylatable mutant (Mut), as substrates. **d**, Phosphoamino-acid analysis of phosphorylated Raf-CR3. Filled ovals are actual signal spots; empty ovals show position of non-radioactive standards. **e**, Two-dimensional tryptic phosphopeptide mapping of phosphorylated Raf-CR3. Thin-layer electrophoresis (TLE) and thin-layer chromatography (TLC) dimensions are indicated. **f**, Two-dimensional map of immunoprecipitated Raf-1 S338/339 phosphopeptides from trypsinized, phosphorylated Raf-CR3 using a rabbit polyclonal antibody raised against Raf-1 residues 337–354.

p37	KELIINEILVM
Pak1	NLQQQPKKELIINEILVMRENKNPNIVNLDVSYLVGDELWVMEYLAGGS
Pak2	NLQQQPKKELIINEILVMRENKNPNIVNLDVSYLVGDELWVMEYLAGGS
Pak3	NLQKQPKKELIINEILVMELKNPNIVNLDVSYLVGDELWVMEYLAGGS
p37	SDNVLLGMEG
Pak1	LTDVVTETCMDEGQIAAVCRECLQALEFLHSNQVIHRDIKSDNITLLGMDG
Pak2	LTDVVTETCMDEGQIAAVCRECLQALEFLHSNQVIHRDIKSDNITLLGMDG
Pak3	LTDVVTETCMDEGQIAAVCRECLQALEFLHSNQVIHRDIKSDNVLLGMEG
p37	SVK
Pak1	SVKLTDFGFCQAITPEQSKRSTMGVTPYWMAPVETRKAYGPKVDIWSLG
Pak2	SVKLTDFGFCQAITPEQSKRSTMGVTPYWMAPVETRKAYGPKVDIWSLG
Pak3	SVKLTDFGFCQAITPEQSKRSTMGVTPYWMAPVETRKAYGPKVDIWSLG
p37	ALYLIATNGTPELQNPKE
Pak1	IMAIEMIEGPPYLNENPLRALYLIATNGTPELQNPKELSAIFRDFLNR
Pak2	IMAIEMIEGPPYLNENPLRALYLIATNGTPELQNPKELSAIFRDFLNR
Pak3	IMAIEMIEGPPYLNENPLRALYLIATNGTPELQNPKELSPIFRDFLNR
p37	ELLQHPFLK
Pak1	LEMDVEKRGSAKELLQHPFLKAKPLSSLTPLTAAAEAKNNH*
Pak2	LEMDVDRRGSAKELLQHPFLKAKPLSSLTPLTAAAEAKNNH*
Pak3	LEMDVEKRGSAKELLQHPFLKAKPLSSLTPLTAAAEAKNNH*

Figure 2 Tryptic peptide microsequencing of p37. Following large-scale purification of the 37K kinase from 40g of rat spleen, four tryptic peptides were analysed by mass-spectrometric microsequence (MSMS) analysis using a Finnigan LCQ Quadrupole Ion Trap Mass Spectrometer. The sequences derived from these four peptides were examined using the SWISS-PROT protein database and all showed absolute identity with Pak3. Interestingly, all of these sequences were located within the catalytic domain of Pak3. Some of the peptides shared identity with other Pak isoforms, but only where they themselves are homologous with Pak3. All sequences shown represent the rat isoforms.

antibodies removed Raf-1 S338 kinase activity in a manner linearly proportional to the amount of protein immunoprecipitated from these samples (data not shown).

Next we determined whether Pak3 regulates Raf-1 activity *in vivo* through direct phosphorylation of S338. We showed previously that constitutive activity of Raf-CX, and of wild-type Raf-1 coexpressed with Ras^{V12}, depends on phosphorylation of S338 and, to a lesser extent, of S339 (ref. 9). The effects of wild-type Pak3 and of dominant-negative Pak3^{R278} (Pak3^{dn}) and constitutively active Pak3^{S91A93A95} (Pak3^{ca}) mutants on the activity of either Raf-CX, or of Raf-1 together with Ras^{V12}, were measured in transient transfections of COS-7 cells (Fig. 4a). Pak3^{R278} reduced both the intrinsic activity of Raf-CX (>70%; Fig. 4a, left) and Ras^{V12}-inducible activation of Raf-1 (>50%; Fig. 4a, centre). Substitution of serines 338 and 339 with aspartic acid and glutamic acid (which mimic phosphorylated residues) results in a Raf-1 protein which retains Ras^{V12}-inducibility⁹. Significantly, Pak3^{R278} failed to inhibit the activation of Raf-1^{D338E339} by Ras^{V12} (Fig. 4a, right). Pak3^{R278} can thus exert its effect only if there are phosphorylatable serine residues at positions 338 and 339 in Raf-1, providing evidence for the direct *in vivo* regulation of Raf-1 by Pak3. Coexpression of wild-type Pak3 or Pak3^{S91A93A95} did not significantly increase the degree of activation of Raf-CX or Ras^{V12}-inducible Raf-1. This has also been shown for Pak1 mutants in a biological assay system¹⁴. In the absence of Ras^{V12}, Pak3^{S91A93A95} stimulated Raf-1 activity to one-third the level of Ras^{V12}-stimulated Raf-1 activity (Fig. 4c). This indicates that Pak activity is not limiting when oncogenic Ras is coexpressed in the cell.

To determine whether Pak3 regulates Raf-1 *in vivo* through phosphorylation of serine 338 or 339, we expressed Pak3^{S91A93A95} together with Raf-1^{A338} or Raf-1^{A339} and measured the ability of Pak3^{S91A93A95} to activate each mutant (Fig. 4c). Pak3^{S91A93A95} was capable of stimulating Raf-1^{A339} but not Raf-1^{A338}, confirming that

Pak3 regulates Raf-1 through S338 directly. Finally, *in vivo* phosphorylation of Raf-1 on S338 by Pak3 was confirmed using antibodies that bind specifically to Raf-1 phosphorylated on S338 (Fig. 4d). Raf-1 coexpressed in COS-7 cells with either Ras^{V12} or Pak3^{S91A93A95} showed, respectively, 2.5- and 6.5-fold increases in phosphoserine 338 levels. This confirmed our earlier report of phosphorylation of Raf-1 S338 *in vivo*⁹.

Phosphorylation of Raf-1 is important for both positive and negative regulation of biological activity⁵. We have isolated and purified Pak3 as one protein kinase responsible for specific positive regulatory phosphorylation of Raf-1 on S338. Pak3 was purified from rat spleen as a truncated protein, probably a result of artefactual cleavage occurring during purification. Interestingly, both Pak1 (ref. 15) and Pak2 (ref. 16) are activated by proteolysis. Phosphorylation of Raf-1 S338 is inducible by Ras^{V12} (ref. 9), but we do not yet know whether the presence of Ras is necessary for Pak3-mediated phosphorylation of Raf-1 or if the localization of Raf-1 to the plasma membrane is sufficient. Constitutively active Pak3 can stimulate the intrinsic activity of a Ras-binding-defective Raf-1 mutant, indicating that physical interaction of Raf-1 with Ras may not be necessary for Raf-1 regulation by Pak3 (data not shown). Pak1 can phosphorylate the kinase Mek1, enhancing the binding of

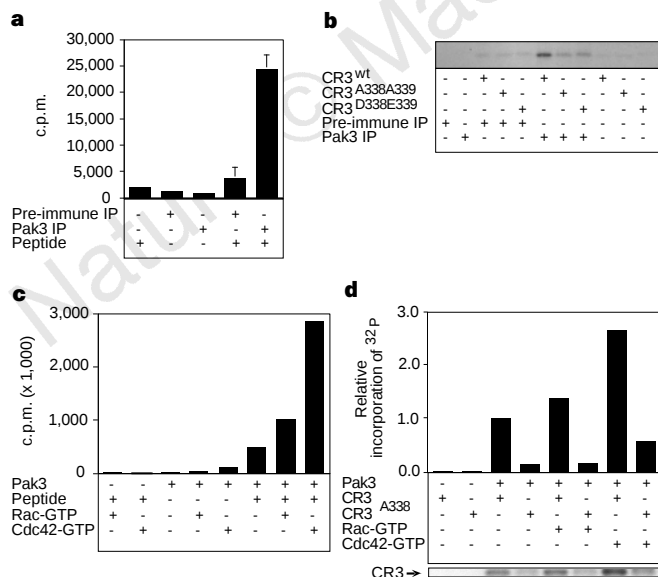


Figure 3 Immunoprecipitated Pak3 and recombinant Pak3 phosphorylated the Raf-1 peptide and Raf-CR3. Pak3 was immunoprecipitated directly from rat spleen lysates using a C-terminal polyclonal antibody (Santa Cruz) and phosphorylated both **a**, the Raf-1(331–345) peptide and **b**, wild-type (wt) Raf-CR3. Specificity was shown by the reduced ability of immunoprecipitated Pak3 to phosphorylate the Raf-CR3^{A338A339} or Raf-CR3^{D338E339} mutant proteins. The antibody used is also weakly crossreactive with Pak1/2 and their potential involvement with Raf-1 activation cannot be completely ruled out. IP, immunoprecipitate. **c**, Phosphorylation of the Raf-1 peptide by recombinant His-tagged mouse Pak3 from Sf9 cells was stimulated in the presence of either Rac-GTPγS or Cdc42-GTPγS. **d**, Wild-type Raf-CR3 but not Raf-CR3^{A338} was phosphorylated by recombinant His-tagged mouse Pak3 which was stimulated by Rac-GTPγS or Cdc42-GTPγS.

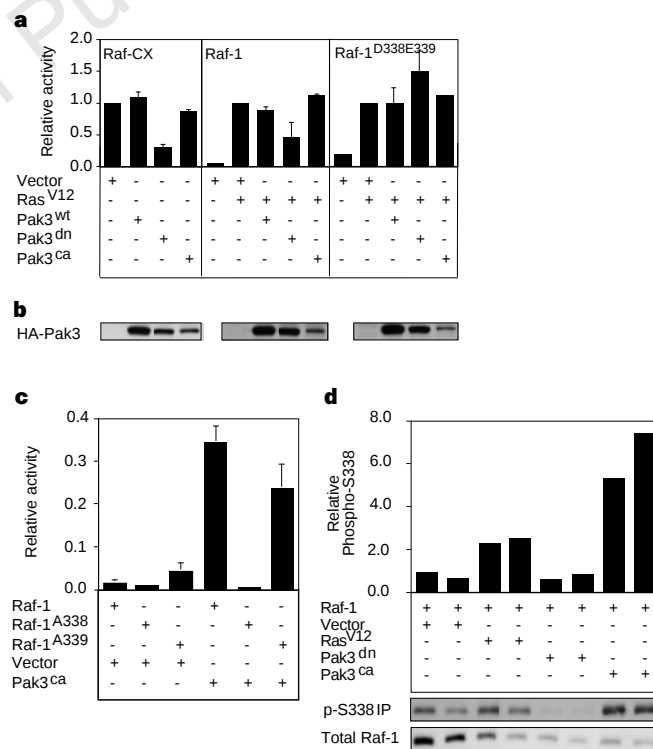


Figure 4 The effect of Pak3 on Raf-1 activation *in vivo* in COS-7 cells. Different forms of haemagglutinin (HA)-tagged Pak3 were transiently transfected together with Raf-1 into COS-7 cells. **a**, The inhibitory effect of dominant-negative Pak3^{R278} (Pak3^{dn}) on the latent activation of Raf-CX is clearly seen as compared with wild-type Pak3 (Pak3^{wt}) and constitutively active Pak3^{S91A93A95} (Pak3^{ca}) (left). Pak3^{dn} inhibited activation of Raf-1 kinase activity in response to oncogenic Ras^{V12} (centre). This result was corroborated by the inability of Pak3^{dn} to inhibit the Ras^{V12}-stimulated activity of the Raf-1^{D338E339} mutant protein (right). **b**, Pak3 expression in **a** was assessed by western blotting. **c**, Pak3^{ca} stimulated the kinase activity of both wild-type Raf-1 and Raf-1^{A339}, but failed to activate the Raf-1^{A338} mutant. Results are normalized to the stimulation of Raf-1 by Ras^{V12}. **d**, Raf-1 phosphorylated on S338 was immunoprecipitated (IP) from COS-7 cell lysates using a phosphoserine-338-specific antibody. Myc-Raf-1 was detected by immunoblotting with the 9E10 antibody and normalized to the total Myc-Raf-1 expressed.

Mek1 to Raf-1 and allowing Pak1 to feed into the Raf pathway at this point¹⁷. We also observed phosphorylation of Mek1 by Pak3 *in vitro* (our unpublished observations). Dominant-negative Pak3^{R278} interferes directly with Raf-1 activation, showing that Pak3 is essential in Raf-1 regulation and, consequently, in MAP kinase signalling.

We are at present studying the ability of Pak1 to phosphorylate and regulate Raf-1, as a dominant-negative Pak1 mutant interferes with transformation of rat-1 fibroblasts by oncogenic Ras¹⁴. We also predict potential regulation of all three mammalian isoforms of Raf by Pak3, as Raf-1 S338 and the amino acids immediately amino-terminal to S338 are conserved in these proteins. We propose that Pak3 represents a point of crosstalk between the Ras/Raf and Pak signalling pathways that may ultimately be vital in the balance between cell survival and apoptosis. Our data lead to the prediction that Cdc42 is a preferred physiological regulator of Pak3, although we do not yet know whether regulation of Raf-1 by Pak3 depends on either Cdc42 or Rac *in vivo*. The Rac guanine-nucleotide-exchange factors Vav and Sos are activated by the Ras-dependent phosphatidylinositol-3-OH kinase^{12,13}. As Vav and Sos control Rac activation and, ultimately, the Pak kinases, the Ras/phosphatidylinositol-3-OH kinase pathway may be capable of exerting broad control over the Ras/Raf pathway. □

Methods

Purification of Pak3 37K fragment from rat spleen. Frozen rat spleens (8–10 g) were homogenized in a low osmotic potential buffer (10 mM Tris-HCl, 50 mM NaF, 5 mM MgCl₂, 1 mM EGTA, 1 mM dithiothreitol (DTT), 250 μM Na₃VO₄, 4 μg ml⁻¹ leupeptin, 4 μg ml⁻¹ pepstatin A, 10 μg ml⁻¹ aprotinin, 10 μg ml⁻¹ soybean trypsin inhibitor, 3 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM benzamidine-HCl, pH 7.5). The extract was made up to 50 mM NaCl and Raf-1 S338/339 kinase was resolved by chromatography over heparin-Agarose, blue-Sepharose, sephacryl Hi Prep S-200, Mono-Q and Mono-P matrices. Extracts were resolved by SDS-PAGE and proteins were visualized by silver staining¹⁸.

Kinase assays. Samples were assessed for their ability to phosphorylate 500 μM Raf-1 peptide (331RPRGQRDSSFFWEIE₃₄₅) in the presence of 500 μM ATP (containing 2 μCi [γ-³²P]ATP) at 30°C for 10 min. Incubations were analysed by P-81 phosphocellulose paper assay. Phosphorylation of S338/339 in Raf-CR3 was determined by similar incubations containing 1 μg substrate at 30°C for 30–60 min. Proteins were resolved by SDS-PAGE and transferred to PVDF, and the dried, Ponceau-S stained membranes were autoradiographed. Raf-1 kinase activity was measured by the coupled assay method¹⁹. In-gel kinase assay was done using the method of ref. 20 with 10% (w/v) acrylamide low-crosslinker gels. In-gel phosphorylation was done using 20 ml assay buffer containing 250 μM ATP and 375 μCi [γ-³²P]ATP at 30°C for 60–90 min. The gel was washed repeatedly, dried and autoradiographed.

Expression of recombinant Raf-CR3 in *Escherichia coli*. DNA sequences encoding residues 307–648 of human c-Raf-1 were amplified by polymerase chain reaction (PCR) using the 5'-primer 5'-GGTGAATTCAGCCGAAAACCCCGTG-3' and the 3'-primer 5'-GCCCTCGAGCTAGAAGACAGGCAGCCTCG-3'. Amplified DNA was purified, digested with *Eco*RI and *Xho*I and cloned into the Stratagene pCAL-n vector. The entire Raf-CR3 coding sequence was verified by termination reaction PCR DNA sequencing. Site mutants were generated using the Promega Altered Sites *in vitro* mutagenesis system. Fusion proteins containing a calmodulin-binding peptide and Raf-CR3 were expressed in *E. coli* strain BL21 (pLysS). Bacteria were induced at 30°C with 0.5 mM isopropyl-β-D-thiogalactoside (IPTG) and Raf-CR3 was recovered using the Stratagene protocol.

Two-dimensional phosphopeptide mapping and phosphoamino-acid analysis. Phosphorylated Raf-CR3 was mapped as described²¹. Phosphopeptides were recovered and electrophoresed on cellulose thin layer plates (Merck 5716) in 1% (w/v) (NH₄)₂CO₃, pH 8.9, at 1,000 V for 25 min. Plates were chromatographed in the second dimension at room temperature for 7.5 h, dried and autoradiographed. Immunoprecipitations from trypsin digests used a rabbit polyclonal antibody raised against human Raf-1 residues 337–354 to identify S338/339-containing phosphopeptides. Peptides were eluted from

beads with pH 1.9 buffer and mapped as above. One-dimensional phospho-amino-acid analysis was performed in pH 3.5 buffer as described²¹.

Transfections. COS-7 cells were transfected with 10 μg DNA by electroporation. Cells were lysed 3 days after transfection and Raf-1 kinase activity was measured¹⁹. Transfection/expression efficiencies were assessed by densitometric scanning of western blots of immunoprecipitates and cell lysates, and the data sets were normalized accordingly.

GTPase activation of kinase activity. GST-Rac1 and GST-Cdc42 (90 μg) were charged with 0.8 mM GTP-γS using 50 μl glutathione-Sepharose beads as described²², except that GTP-γS binding was achieved by incubation for 25 min at 30°C. Proteins were eluted from beads with buffer containing 100 mM glutathione, pH 7.5, at 4°C for 15 min. Each 10 μl kinase sample was then incubated with 5 μg activated GTPase at 4°C for 60 min before kinase assay as described above.

Immunological detection of Raf-1 containing phosphoserine 338. Rabbits were immunized with the KLH-conjugated phosphopeptide RPRGQRDpSSYYWEI. Antibodies from pooled bleeds were affinity-purified over a phosphopeptide column and then passed over a non-phosphorylated-peptide column to remove antibodies specific for the non-phosphorylated peptide. The purified antibody (6 μg) was used to immunoprecipitate Raf-1 phosphorylated on S338 from transfected COS-7 cell lysates (100 μg). Immune complexes were captured on protein-A-Sepharose beads, washed with lysis buffer and solubilized in SDS-PAGE sample buffer. Immunoprecipitates from equal amounts of lysate (60 μg) were resolved on 8% (w/v) acrylamide gels and proteins were transferred to PVDF membranes. Myc-Raf-1 present in each immunoprecipitate, corresponding to the S338-phosphorylated population, was visualized with the anti-Myc-epitope antibody 9E10. Relative expression levels of Myc-Raf-1 were determined by analysing equal amounts of total lysate protein from each transfection using the antibody 9E10.

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Correspondence and requests for materials should be addressed to M.S.M. (e-mail: mmarsha@iupui.edu).

Histone acetyltransferase activity of CBP is controlled by cycle-dependent kinases and oncoprotein E1A

S. Ait-Si-Ali*, S. Ramirez*, F.-X. Barre*, F. Dkhissi*, L. Magnaghi-Jaulin*, J. A. Girault†, P. Robin*, M. Knibiehler‡, L. L. Pritchard*, B. Ducommun‡, D. Trouche* & A. Harel-Bellan*

*Laboratoire Oncogénèse, Différenciation et Transduction du Signal, CNRS UPR 9079, IFC-01, 94801 Villejuif, France

†INSERM U114, Collège de France, 75005 Paris, France

‡IPBS, CNRS UPR 9062, Université Paul Sabatier, 31077 Toulouse, France

Transforming viral proteins such as E1A force cells through the restriction point of the cell cycle into S phase by forming complexes with two cellular proteins^{1–3}; the retinoblastoma protein (Rb)⁴, a transcriptional co-repressor⁵, and CBP/p300 (ref. 6), a transcriptional co-activator^{7–9}. These two proteins locally influence chromatin structure: Rb recruits a histone deacetylase^{10–12}, whereas CBP is a histone acetyltransferase^{13,14}. Progression through the restriction point is triggered by phosphorylation of Rb, leading to disruption of Rb-associated repressive complexes and allowing the activation of S-phase genes¹⁵. Here we show that CBP, like Rb, is controlled by phosphorylation at the G1/S boundary, increasing its histone acetyltransferase activity. This enzymatic activation is mimicked by E1A.

CBP histone acetyltransferase (HAT) activity was measured in cells synchronized by serum starvation and after stimulation by serum for various times (Fig. 1a). HAT activity remained stable during the first hours of the cell cycle but increased sharply at 9–10 hours after addition of serum, with a peak around the G1/S transition. This induced HAT activity did not correlate with accumulation of the protein (Fig. 1b), indicating that it could be due to post-translational modification, for example by phosphorylation. p300, a protein highly homologous to CBP, is a phosphoprotein^{16,17} whose phosphorylation is regulated by external stimuli¹⁶ in a cell-cycle-dependent manner¹⁷. We therefore investigated whether phosphorylation of CBP could induce its enzymatic activation.

Measurements of ³²P incorporation at different times after serum stimulation confirmed that CBP is a phosphoprotein (Fig. 1b), and that it is hyperphosphorylated when CBP HAT activity is maximal (Fig. 1b). Phosphorylation and HAT activity both returned to basal levels later in the cycle (Fig. 1b). The principal kinase activated before the G1/S transition is the cyclin E–Cdk2 complex, which binds CBP¹⁸. The peak of CBP HAT enzymatic activity coincided with the peak of cyclin E–Cdk2 activity (Fig. 1c). Furthermore, roscovitine, an inhibitor of the cyclin E–Cdk2 complex, blocked both the phosphorylation and the enzymatic activation of CBP (Fig. 1d), suggesting that Cdk2 is involved in CBP phosphorylation at the G1/S boundary. *In vitro* experiments using purified recombinant proteins indicated that cyclin E–Cdk2 complexes can specifically phosphorylate CBP and not equivalent amounts (data not shown) of irrelevant proteins (Fig. 2b). Analysis of CBP deletion mutants (Fig. 2a, b) suggested that CBP phosphorylation by cyclin E–Cdk2 occurs in the carboxy-terminal region of the protein, outside the HAT domain. Treatment of recombinant CBP with purified cyclin E–Cdk2 *in vitro* also caused HAT activity to increase (Fig. 2c); no HAT activity was detectable in the cyclin E–Cdk2 sample (data not shown). Treatment of phosphorylated samples with phosphatase abolished the enzymatic activation (Fig. 2d), confirming that activation was due to phosphorylation of CBP. Furthermore, enzymatic activation and phosphorylation correlated

well: mutants that have lost the C-terminal part of the molecule, a region that in isolation is heavily phosphorylated (Fig. 2b), are neither phosphorylated nor activated by cyclin E–Cdk2 (Fig. 2e). Finally, *in vitro*, CBP was a better substrate for cyclin E–Cdk2 than for cyclin A–Cdk2, another kinase that is activated at the G1/S boundary; and cyclin A–Cdk2 did not affect CBP HAT activity (data not shown). Preliminary results indicated that the ratio of serine-to-threonine phosphorylation was 2:1 for both CBP immunoprecipitated from cell extracts and CBP phosphorylated *in vitro* (our unpublished observations), but their two-dimensional tryptic maps were different. Thus, CBP may not be phosphorylated by cyclin E–Cdk2 in live cells, but by a distinct kinase. Alternatively, this discrepancy may result from additional post-translational modifications, which might also be involved in CBP regulation. Taken together, our results show that CBP HAT enzymatic activity is regulated in a cell-cycle-dependent manner and that phosphorylation of the protein by a cycle-dependent kinase is involved in this regulation.

CBP is an obligatory target for transforming viral proteins, such as E1A from adenovirus³, that interact with the protein⁷. E1A, like the cyclin E–Cdk2 complex, triggers progression into S phase, so we tested the effect of E1A on CBP HAT activity. CBP HAT activity was higher in stable transfectant cells expressing E1A than in control cells lacking E1A (Fig. 3a, b). The effect was modest in exponentially growing cells (Fig. 3a), in which the amount of CBP detected was consistently lower, but was dramatic (Fig. 3b) in cells arrested in G1 by serum starvation (data not shown). Although E1A induces

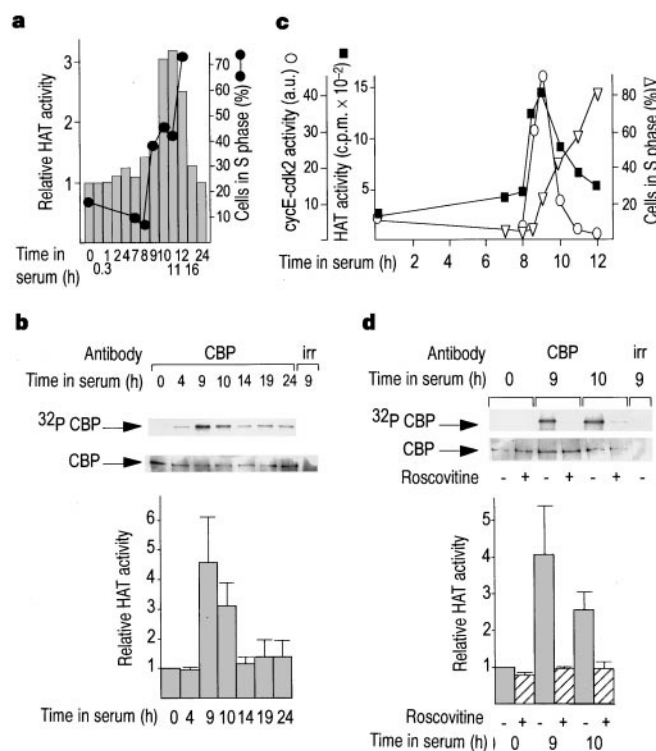


Figure 1 CBP HAT activity is modulated in a cell-cycle-dependent manner. **a**, CBP HAT activity through the cell cycle. Cell-cycle phase of synchronized NIH3T3 cells was determined by flow cytometry; in parallel, CBP was immunoprecipitated and assayed for HAT activity. **b**, Increased HAT activity of CBP correlates with increased phosphorylation: autoradiography (top panel), western blot analysis (middle) and HAT activities (bottom) of immunoprecipitates from cells incubated with ³²P inorganic phosphate. Longer exposures revealed phosphorylation of CBP in quiescent cells (not shown). **c**, CBP HAT activity increases concomitantly with cyclin E–Cdk2 activity, assayed on H1 histone (Sigma). **d**, Roscovitine blocks CBP HAT activation: synchronized NIH3T3 cells were treated with serum together with roscovitine (60 μM) and tested as in **b**.

phosphorylation of p300 in infected cells¹⁶, CBP phosphorylation was not modified in the stable transfectants (data not shown), suggesting that the effect of E1A on CBP HAT activity is direct and due to interaction between E1A and CBP⁷. This was confirmed *in vitro* by using recombinant purified proteins. Incubation of bacterially produced CBP with bacterially produced E1A (which does not have any detectable intrinsic HAT activity; data not shown) induced CBP HAT enzymatic activity in a dose-dependent manner (Fig. 3c). This activation was specific and was not observed with equivalent amounts (data not shown) of various other proteins (Fig. 3d), including TFIIB which interacts with CBP (Fig. 3e). In addition, a CBP mutant that has lost the interaction domain for E1A (CBP1-1696) does not respond to the transforming protein (Fig. 3f). Together, these results indicate that E1A activates the CBP HAT enzyme by binding to it. It is likely that the interaction with E1A, which occurs in a region near the HAT domain, induces a conformational change in this domain which leads to increased catalytic activity. A similar conformational change would be induced by cyclin E-Cdk2 through phosphorylation of the C-terminal region of CBP. As phosphorylation by cyclin E-Cdk2 (Fig. 2b) and interaction with E1A (ref. 7) both occur outside the HAT domain, these results suggest that CBP HAT activity is controlled by an intramolecular regulatory domain. Interaction with E1A and phosphorylation by cyclin E-Cdk2 occur in distinct parts of the molecule but have similar effects on CBP HAT activity, probably by inducing a similar change of conformation.

Our data fit a model in which phosphorylation of CBP by cyclin E-Cdk2, or by an as-yet uncharacterized kinase, stimulates its HAT activity at the G1/S boundary in normal cells, whereas it is constitutively active in some transformed cells as a result, for example, of its association with transforming proteins such as E1A or, as in some leukaemias, of its fusion with other proteins¹⁹.

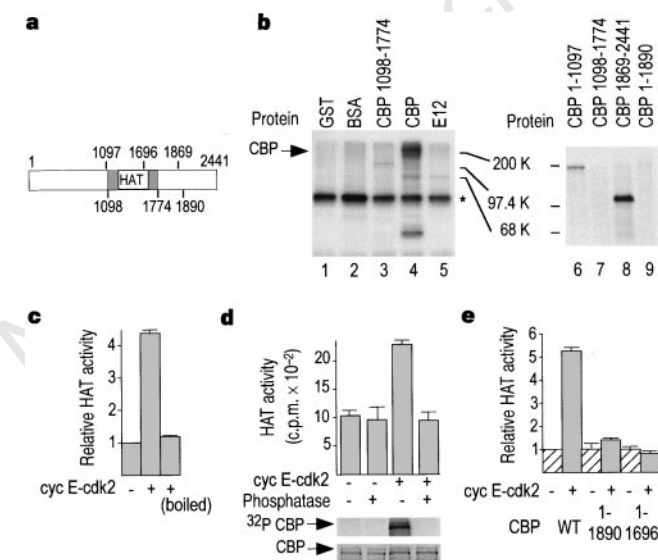


Figure 2 Phosphorylation of CBP stimulates its HAT activity. **a**, Diagram of CBP indicating endpoints of deletion mutants. **b**, CBP is phosphorylated by cyclin E-Cdk2 in the C-terminal region. A fusion protein of glutathione-S-transferase with CBP (GST-CBP) or deletion mutants was incubated with purified cyclin E-Cdk2 in the presence of [γ -³²P]ATP and analysed by SDS-PAGE. Asterisk, cyclin E. All proteins shown, except BSA and GST, consist of fusions of GST with the indicated protein. **c**, *In vitro* treatment of CBP by cyclin E-Cdk2 activates CBP HAT activity. GST-CBP was treated with cyclin E-Cdk2 and assayed for HAT activity. **d**, Induction of CBP HAT activity is due to phosphorylation. GST-CBP was treated with cyclin E-Cdk2 and/or phosphatase. Aliquots were tested for HAT activity or were resolved on SDS-PAGE, followed by staining with Coomassie blue and autoradiography. **e**, CBP HAT activation correlates with phosphorylation. Wild-type (WT) CBP or deletion mutants were treated with cyclin E-Cdk2 and assayed for HAT activity as in **c**.

The purpose of CBP enzymatic activation at the G1/S boundary is not known: it may play a role in some cellular processes, for example in DNA replication, but we consider that it influences CBP's ability to activate transcription, for example of S-phase genes (Fig. 4). During early G1, S-phase genes would be repressed by a complex that includes pocket proteins and histone deacetylases. Cyclin-CDK(s) could trigger their derepression by phosphorylating Rb and, concomitantly, they and/or other kinases could phosphorylate and activate CBP. Note that CBP is a co-activator for E2F (ref. 20), a key factor for S-phase gene induction that is also involved in the activation of the adenovirus early promoter E2 and which is a target for Rb²¹.

CBP is involved in transactivation by a variety of transcription factors²² that have opposite effects on the cell, some being associated with cell proliferation (such as E2F (ref. 20)) and others (such as the myogenic differentiation factors²³) with cell differentiation. Progression into S phase requires the activation of only the proliferation-associated gene subset. CBP HAT activity is not involved in the transcription of all of the CBP-dependent genes²⁴⁻²⁷, and it may be significant that myogenic differentiation factors and retinoic acid receptors, which both inhibit proliferation, do not use the CBP HAT domain to activate their target genes^{24,25} and so would be insensitive to activation of CBP at the G1/S boundary. Only proliferation-associated genes would be activated by the concomitant inactivation of Rb and activation of CBP; E1A would mimic both processes. Several reports describe repression of p300 by E1A (see ref. 9, for example), contrasting with the activation described here. CBP has several activation domains and the domain used varies from one promoter to another. Furthermore, the transactivation capability of its HAT domain is also very promoter-dependent²⁸. The measured effect of E1A on CBP activity is therefore likely to depend on the system analysed and the transactivation domain involved. □

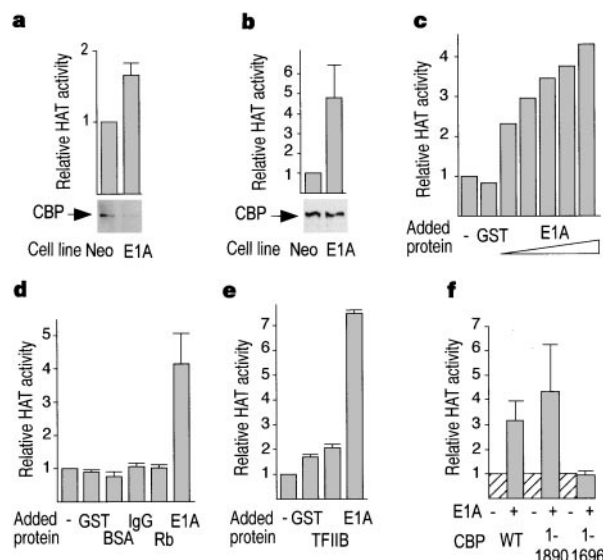


Figure 3 E1A mimics the effect of phosphorylation on CBP HAT activity. **a**, *In vivo* expression of E1A in non-synchronized, cycling cells (**a**) or serum-starved cells (**b**) increases CBP HAT activity. HAT activity (top panel) or western blotting (bottom panel) of CBP immunoprecipitated from stable transfectants expressing E1A (E1A) or not (Neo). **c-e**, E1A activates CBP HAT activity *in vitro*. GST-CBP was incubated for 10 min with GST-E1A (**c**) or with similar amounts of various proteins (Rb:His-Rb) (**d**, **e**). The labels E1A and TFIIB in **c-e** indicate fusion proteins containing GST and either E1A or TFIIB. **f**, CBP HAT activation by E1A correlates with direct interaction. Wild-type (WT) CBP or deletion mutants were treated with E1A and assayed for HAT activity as in **c**.

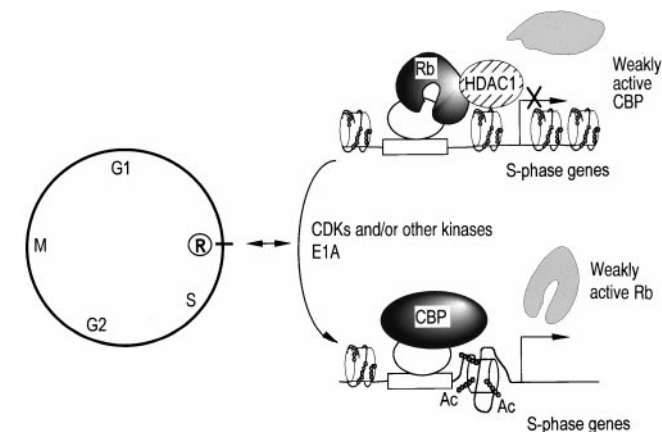


Figure 4 Hypothetical function of CBP HAT activation near the G1/S transition. At the restriction point (R), Rb is inactivated. Concomitantly, CBP is phosphorylated and activated; both actions are mimicked by E1A in transformed cells. Target genes go from a repressed state, in which the chromatin has a closed structure due to the effects of histone deacetylases recruited to the promoter by Rb, to an active state, in which the chromatin has an open configuration due to the histone acetyltransferase activity of CBP; acetylated core histone tails are designated by an Ac tag.

Methods

Plasmids. pGST-CBP: a *Bam*HI insert from pRSV CBP (gift from R. Goodman) was inserted in-frame into pGEX-2TK. A stop codon was corrected by replacing the *Afl*III–*Bst*107 fragment with the corresponding sequence from pBSFL2CBP (gift from T. Kouzarides). Details of other constructions are available on request.

Metabolic labelling. NIH3T3 cells were stably transfected with pRSV-E1A 12s (E1A)²⁰ or a neomycin control vector and assayed for E1A expression (data not shown). For metabolic labelling, 10⁶ NIH3T3 cells were incubated for 4 h with 0.5 mCi ml⁻¹ ³²P inorganic phosphate (Amersham) in phosphate-free medium (Sigma) before immunoprecipitation of CBP.

Immunoprecipitation and western blotting. Stringent immunoprecipitations were performed using standard procedures; cells were lysed in RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% N-P40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA). SRC-1 and P/CAF were undetectable in the immunoprecipitates (data not shown). Mild immunoprecipitation was performed as described¹⁰. All buffers contained protease (Boehringer) and phosphatase (Sigma) inhibitors. Western blotting was done using standard procedures and visualized using an ECL⁺ kit (Amersham). Anti-CBP antibodies used were the A22 antibody (Santa Cruz) for immunoprecipitation and the NM11 antibody (Pharmingen) for western blotting.

In vitro phosphorylation and phosphatase treatment. Cyclin E/Cdk2 protein kinase, expressed in baculovirus-infected Sf9 cells, was purified by standard chromatographic procedures. GST proteins were purified as described²⁹ and dialysed against TBS-G (20 mM Tris, pH 8.0, 150 mM NaCl, 10% glycerol). GST-CBP contained full-length CBP (as assessed by western blot analysis with anti-C-terminus and anti-N-terminus antibodies; data not shown). His-Rb was prepared using the Qiagen protocol. Recombinant proteins were *in vitro* phosphorylated by incubation with 50 ng cyclin E-Cdk2, 100 μM ATP and [³²P]ATP (100 mCi mmol⁻¹ final specific activity) for 45 min at 30 °C in 30 μl buffer containing 25 mM Tris, pH 7.5, 0.1 mM NaVO₄, 0.1 mM EGTA, 10 mM magnesium acetate, 0.04 mM DTT, 0.1 mM ZnSO₄ and protease inhibitors. Phosphatase treatment was performed using 400 U of lambda protein phosphatase (Biolabs) for 30 min at 30 °C.

HAT assay. HAT assays³⁰ were done using a synthetic peptide (Chiron) corresponding to the first 24 amino acids of histone H4 coupled through a linker sequence to a biotin molecule.

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Correspondence and requests for materials should be addressed to A.H.-B. (e-mail: ahbellan@vjfr.cnrs.fr).

Crystal structure of a bacterial signal peptidase in complex with a β-lactam inhibitor

Mark Paetzel^{*}, Ross E. Dalbey[†] & Natalie C. J. Strynadka^{*}

^{*} Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver V6T 1Z3, British Columbia, Canada

[†] Department of Chemistry, The Ohio State University, Columbus, Ohio 43210, USA

The signal peptidase (SPase) from *Escherichia coli* is a membrane-bound endopeptidase with two amino-terminal transmembrane segments and a carboxy-terminal catalytic region which resides in the periplasmic space¹. SPase functions to release proteins that have been translocated into the inner membrane from the cell

interior, by cleaving off their signal peptides¹. We report here the X-ray crystal structure of a catalytically active soluble fragment of *E. coli* SPase (SPase $\Delta 2-75$)^{2,3}. We have determined this structure at 1.9 Å resolution in a complex with an inhibitor, a β -lactam (5S,6S penem)^{4,5}, which is covalently bound as an acyl-enzyme intermediate to the γ -oxygen of a serine residue at position 90, demonstrating that this residue acts as the nucleophile in the hydrolytic mechanism of signal-peptide cleavage. The structure is consistent with the use by SPase of Lys 145 as a general base in the activation of the nucleophilic Ser 90, explains the specificity requirement at the signal-peptide cleavage site, and reveals a large exposed hydrophobic surface which could be a site for an intimate association with the membrane. As enzymes that are essential for cell viability, bacterial SPases present a feasible antibacterial target⁴⁻⁶: our determination of the SPase structure therefore provides a template for the rational design of antibiotic compounds.

The *E. coli* SPase $\Delta 2-75$ structure has a mainly β -sheet protein fold, consisting of two large antiparallel β -sheet domains (termed I and II and coloured green and blue, respectively, in Fig. 1), two small 3_{10} -helices (consisting of residues 246–250 and 315–319), and one small α -helix (residues 280–285). There is one disulphide bond, as was found in earlier biochemical studies⁷, between Cys 170 and Cys 176. This bond is located immediately before a β -turn in the

domain II β -sheet (Fig. 1). In addition, an extended β -ribbon (residues 107–122, coloured purple in Fig. 1) protrudes from domain I, together with the N-terminal strand, giving the SPase $\Delta 2-75$ molecule an overall conical shape with rough dimensions of $60 \times 40 \times 70$ Å (Figs 1, 2).

Sequence alignments indicate that highly conserved regions of primary sequence within the prokaryotic and eukaryotic SPases¹ reside within domain I of the *E. coli* SPase structure, whereas the two insertions representing the extended β -ribbon (residues 107–122) and domain II are variably present from species to species. In addition, domain I shares structural similarities with UmuD' protease⁸, the proteolytic domain of a self-cleaving repressor protein involved in the 'SOS' DNA-repair response in *E. coli*. Although the overall mainchain connectivity in UmuD' and domain I of SPase differs in some regions, 68 common C α atoms can be superimposed with a root mean square (r.m.s.) deviation of 1.6 Å. Domain II and the extended β -ribbon of SPase have no structural counterparts in UmuD'. Domain I, containing all of the essential and conserved catalytic elements, represents a new protease structural motif that is likely to be conserved from bacteria to man.

A large, unusually exposed hydrophobic surface extends across the SPase $\Delta 2-75$ molecule and includes the substrate-binding site and catalytic centre (labelled S1, S3 and Ser 90 in Fig. 2). The residues contributing to the hydrophobic character of this surface

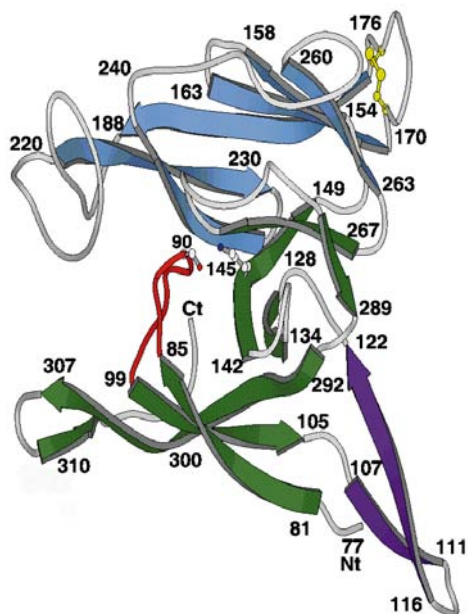


Figure 1 A ribbon diagram²⁸ showing the general fold of SPase $\Delta 2-75$. The domain I β -sheet (the conserved catalytic core) is shown in green and the domain II β -sheet in blue. The β -hairpin extension protruding from domain I is shown in purple. The active-site residues Ser 90 and Lys 145 are labelled 90 and 145. The loop containing the nucleophilic Ser 90 is in red. The disulphide bond between Cys 170 and Cys 176 is shown in yellow. The inhibitor is not shown for clarity. Likewise, some small β -strands and helices are shown as random coils for clarity. The antiparallel β -strands consisting of residues 81–85, 99–105, 292–307 and 312–314 form a large exposed hydrophobic surface (the proposed membrane-association surface).

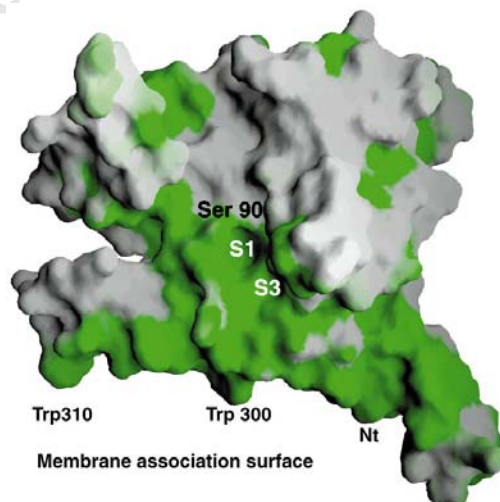


Figure 2 A GRASP²⁹ representation of the molecular surface of SPase $\Delta 2-75$. The view is the same as in Fig. 1. Green represents exposed hydrophobic surfaces. The substrate-binding sites S1 and S3 are labelled, as is Ser 90 of the active site. Trp 300, Trp 310, and the N terminus (Nt) are labelled along the large hydrophobic surface, the proposed membrane-association surface.

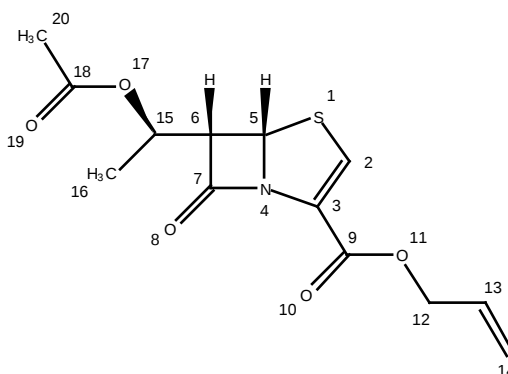


Figure 3 Structure of the β -lactam-type inhibitor allyl (5S,6S)-6-[(R)-acetoxyethyl]-penem-3-carboxylate^{4,5}.

protrude from the main β -sheet of domain I and include Phe 79, Ile 80, Tyr 81, Phe 100, Leu 102, Trp 300, Met 301, Phe 303, Trp 310, Leu 314, Leu 316, and Ile 319. On the basis of our observations of the structure we suggest that, *in vivo*, the membrane-anchored N-terminal strand and the associated β -ribbon, from residues 106 to 124 (Fig. 1), would bend in the appropriate manner to allow the exposed hydrophobic surface of SPase to insert into the membrane lipid bilayer, presumably optimizing contact with the signal-peptide cleavage site. This proposal is consistent with earlier experiments that showed that the detergent Triton-X100 is essential for optimal activity of SPase $\Delta 2-75$ (ref. 3), as well as for optimal growth of the SPase $\Delta 2-75$ crystals⁹. Recent biophysical studies¹⁰ have revealed that SPase $\Delta 2-75$ inserts into the outer leaflet of the *E. coli* inner membrane. In addition, it has been suggested¹¹ that Trp 300 and Trp 310 (Figs 1, 2) are essential for the catalytic activity of *E. coli* SPase. This result is intriguing given the distance (>20 Å) between these residues and the active site and their location on the hydrophobic surface, the proposed membrane-association surface (Figs 1, 2). Tryptophans and other aromatic residues are commonly found at membrane-protein interfaces¹².

Although bacterial SPases are not inhibited by standard protease inhibitors, they are inhibited by β -lactam compounds with 5S stereochemistry^{4,5}. We have determined the structure of SPase $\Delta 2-75$ in the presence of a 5S,6S β -lactam (penem), an SPase inhibitor (Fig. 3)^{4,5}. The electron density shows a covalent bond between SPase Ser 90 O γ and the carbonyl carbon (C7) of the inhibitor, with the four-membered β -lactam ring being cleaved between C7 and N4 (Fig. 4). This is, to our knowledge, the first direct evidence for the role of Ser 90 O γ as the acylating nucleophile in catalysis. The structure shows that the Ser 90 O γ attacks the *si*-face of the β -lactam amide bond, a peptide-bond analogue. This indicates that SPase may be unique among serine-dependent hydrolases, including the serine proteases^{4,13} and the group 2b β -lactamases¹⁴, which prefer a *re*-face attack. A *si*-face nucleophilic attack by *E. coli* SPase was predicted previously on the basis of stereochemical requirements of several inhibitory compounds⁴.

The main-chain amide of Ser 90 forms a strong hydrogen bond

(of length 2.9 Å) with the carbonyl oxygen (O8) of the cleaved β -lactam ring (Fig. 4). This indicates that the Ser 90 amide might contribute to the formation of an 'oxanion hole', lending electrophilic assistance by stabilizing the tetrahedral transition-state intermediate. There appears to be no suitably positioned second main-chain or sidechain amide that could contribute to the oxanion hole (as is found in the group 2b β -lactamases¹⁴ and the serine proteinases¹⁵). However, the Ser 88 side chain could potentially participate in such an interaction by a simple rotation from the observed χ_1 of -54° (Fig. 4) to a value of $+60^\circ$ (Fig. 5). This interaction is prevented in the inhibitor complex by an unfavourable van der Waals contact between the Ser 88 O γ in the $+60^\circ$ conformation and the S1 and C15 atoms of the inhibitor (Fig. 4). The Ser 88 side chain has the highest temperature factors in the active-site region, indicating that it is not in an optimal environment in the inhibitor complex. The contribution of a serine hydroxyl to an oxanion hole has been seen previously in lipolytic enzymes such as cutinase¹⁶.

The Lys 145 N ζ position is fixed relative to Ser 90 O γ by hydrogen bonds to Ser 278 O γ (bond length 2.9 Å) and to the carbonyl oxygen (O10) of the inhibitor side chain (bond length 2.9 Å) (Fig. 4). The N ζ of Lys 145 is 2.9 Å away from the Ser 90 O γ and is the only titratable group in the vicinity of the active-site nucleophile (Fig. 4). The next closest ionizable group, 7.5 Å away from Ser 90 O γ , is Asp 280 which is held in place by a strong salt bridge to Arg 282 (Fig. 4). Thus, the ϵ -amino group of Lys 145 is suitably positioned to act as the general base in both acylation and deacylation steps of catalysis. It appears as though the inhibitor has displaced the deacylating water, as no water molecules are found within 5.5 Å of the covalent inhibitor link. As the co-crystals containing enzyme and inhibitor were grown weeks before the data collection, the acyl-enzyme must be extremely stable, supporting the idea that a deacylating water molecule is displaced. The side chain of Lys 145 is completely buried in this inhibitor complex (Fig. 4), in which it makes van der Waals contacts with the sidechain atoms of Tyr 143, Phe 133 and Met 270, and with the main-chain atoms of Met 270, Met 271, Gly 272 and Ala 279, all of which come from domain I. The

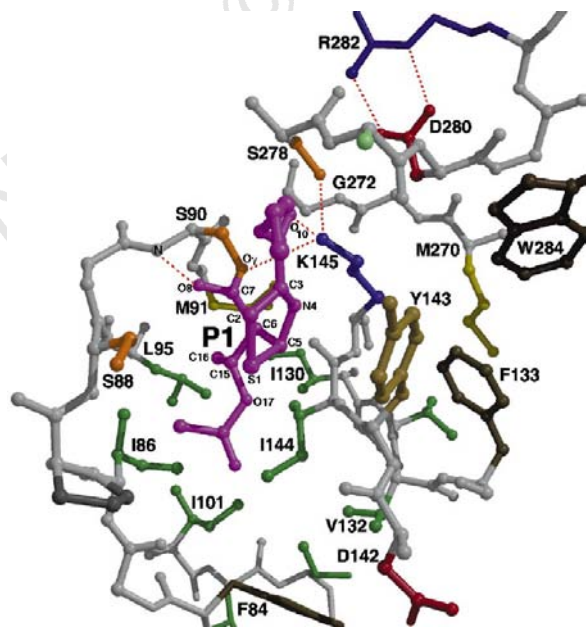


Figure 4 A ball-and-stick representation³⁰ of the active-site residues of SPase $\Delta 2-75$. The β -lactam (5S,6S penem) inhibitor^{4,5} shown in Fig. 3 and in this figure (purple) is covalently bound to the O γ of Ser 90, with the carbonyl oxygen (O8) of the cleaved β -lactam (the bond between C7 and N4 has been cleaved) sitting in the oxanion hole formed by the main-chain nitrogen of Ser 90 (S90). The methyl group (C16) of the inhibitor, labelled P1, sits in the S1 substrate-binding site.

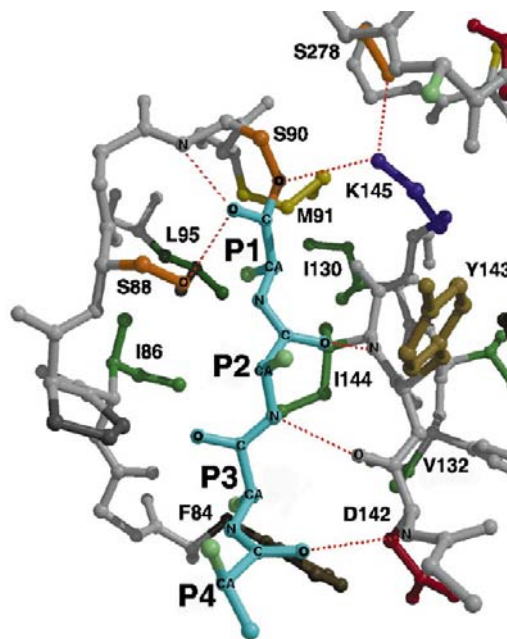


Figure 5 A ball-and-stick representation³⁰ of the active-site residues of SPase $\Delta 2-75$ with the P1-P4 residues of an acylated peptide substrate (Ala-Ala-Ala-Ala) modelled into the binding sites S1-S4. The observed positions of the methyl group (C16) and the carbonyl oxygen (O8) of the inhibitor (Fig. 4) were used as a guide.

hydrophobic environment surrounding the Lys 145 ϵ -amino group is probably essential for lowering its pK_a so that it can stay in the deprotonated state required for its function as the general base^{17,18}.

A typical *E. coli* signal peptide consists of a positively charged N terminus, an inner hydrophobic core, and a C-terminal cleavage-recognition sequence containing small uncharged residues at the P1 (–1) and P3 (–3) sites^{19,20}. Alanine residues are the most common residues at the –1 and –3 positions, giving rise to the so-called –1, –3 or Ala-X-Ala, rule^{19,20}. The sidechain methyl group (C16) of the penem is located in the SPase substrate-binding pocket (S1) (Figs 2, 4). This methyl group is essential for the effectiveness of the inhibitor^{4,5} and probably mimics the P1 (–1) (Ala) side chain of the substrate. The residues making direct van der Waals contacts with the P1 methyl group in the S1 specificity pocket are Met 91, Ile 144, Leu 95 and Ile 86 (Fig. 4).

Using the position of the inhibitor methyl group (C16) in the S1 site and of the inhibitor carbonyl group (C7, O8) in the oxyanion hole as a guide, we have modelled a tetrapeptide (poly-Ala) into the active site of SPase (Fig. 5). We needed an extended, β -strand conformation of the peptide substrate to provide both a favourable fit and β -sheet-type hydrogen bonds with the conserved β -strand containing Lys 145, supporting earlier studies which indicated that the C-terminal five to six residues of the signal peptide would adopt a β -sheet conformation²⁰. This model helps to explain the cleavage-site specificity of SPase. The side chain of the P1 Ala occupies the same site as the inhibitor methyl group and the side chain of the P3 Ala points into a shallow hydrophobic depression formed by Phe 84, Ile 86, Ile 101, Val 132, Ile 144 and the C β of Asp 142 (the proposed substrate-specificity site S3; Figs 2, 5). Although alanine is the most common residue at the P3 site of signal peptides, larger aliphatic residues such as Val, Leu and Ile can also occur at this position. Our structure shows that the hydrophobic depression for the S3 site is broader than that for the S1 site (Figs 2, 5). The S3 site could therefore accommodate these larger residues at the P3 site of the signal-peptide substrate. The side chains of the residues at P2 and P4 point out of the active site towards the solvent (Fig. 5), consistent with the observed signal-peptide sequence variability at these positions¹⁹.

Future modelling studies aimed at an understanding of the structure and function of the eukaryotic SPases will proceed on

the basis of the conservation of primary sequence¹ within the *E. coli* SPase domain I, the catalytic core of type 1 SPases. Important issues, such as the reasons behind unique substrate specificity of mitochondrial SPases¹ and the substitution of the catalytic lysine by the more typical histidine in the endoplasmic reticulum SPases¹, can now be addressed from this first structure of an SPase. □

Methods

Data collection. The SPase $\Delta 2$ –75 protein (relative molecular mass (M_r) 27,952; 249 amino-acid residues) was expressed and purified as described⁹. The crystals were grown in the presence of the inhibitor allyl (5S,6S)-6-[(R)-acetoxyethyl]penem-3-carboxylate^{4,5} and the detergent Triton-X100. Because of the complicated methodology involved, the procedure for the crystallization of the orthorhombic crystal form of SPase $\Delta 2$ –75 will be published elsewhere. The crystals belong to the orthorhombic space group $P2_12_12$ with unit-cell dimensions of $a = 110.7 \text{ \AA}$, $b = 113.2 \text{ \AA}$, $c = 99.2 \text{ \AA}$. The specific volume (V_m)²¹ of the crystals was $2.78 \text{ \AA}^3 \text{ Da}^{-1}$ for four molecules in the asymmetric unit. The fraction of crystal volume occupied by solvent was $\sim 56\%$. The ethylmercury phosphate and methylmercury acetate soaks were done at concentrations of 4.9 mM and 6.1 mM for 6 and 12 h, respectively. The diffraction intensities were measured at 100 K on beamline X12C at the Brookhaven National Synchrotron Light Source (NSLS). The data were processed with the program DENZO²².

Phase determination and refinement. We determined the crystal structure of SPase $\Delta 2$ –75 by multiple isomorphous replacement with anomalous signal (MIRAS) using the phases calculated from two heavy-atom derivatives (ethylmercury phosphate and methylmercury acetate)²³. The heavy-atom parameters were refined and phases calculated using the program MLPHARE²³. The resulting electron-density map was greatly improved by solvent flattening, histogram matching, and non-crystallographic symmetry averaging (four molecules in the asymmetric unit) using the program DM²³. Molecular-model building into the electron-density map was done with the program O (ref. 24) and the structure was refined using the programs XPLOR²⁵ and TNT²⁶. Phasing and refinement statistical parameters are shown in Table 1. The most disordered regions in each of the molecules in the asymmetric unit are extended loops or hairpins near the solvent surface (residues 108–124, 170–176, 198–202 and 304–313) and are still under refinement. The N-terminal Met and Val 76 are not observed in any of the four molecules of the asymmetric unit. An error in the reported amino-acid sequence²⁷ was observed from the electron density and confirmed by DNA sequencing: Ala (GCT) 182 is Val (GTC) 182.

Table 1 Crystallographic data

Data collection							
Data set	d_{max}^* (Å)	Reflections			$\langle I/\sigma(I) \rangle$	$R_{\text{merge}}^{\dagger}$ (%)	
		Total observed	Unique	Percent of possible			
Native	1.9	391,951	88,159	97.2	21.9	5.6	
Ethylmercury phosphate	2.9	98,274	28,379	99.6	7.9	6.9	
Methylmercury acetate	2.9	91,557	21,851	76.5	6.8	10.3	
Phasing statistics							
Derivative	Resolution (Å)	Sites		PhP‡ Acentric/centric	$R_{\text{cullis}}^{\S}$ Acentric/centric		
Ethylmercury phosphate	20.0–2.9	7		1.30/1.02	0.78/0.72		
Methylmercury acetate	10.0–4.0	2		0.95/0.69	0.86/0.84		
Current refinement statistics							
Completeness of model			$R_{\text{f}}^{\P}$ (%)	$R_{\text{free}}^{\#}$ (%)	r.m.s. deviation		$B_{\text{ave}}^{\&}$ (Å ²)
Residues	Atoms	Water molecules			Bonds (Å)	Angles (°)	
988	7,899	253	22.5	27.7	0.016	1.9	29.2

* d_{max} is the maximum resolution of measured X-ray intensities.

$\dagger R_{\text{merge}} = \sum_i |I_{\text{obs},i} - \langle I_{\text{ave},i} \rangle| / \sum_i I_{\text{ave},i}$, where $\langle I_{\text{ave},i} \rangle$ is the average structure-factor amplitude of reflection i and $I_{\text{obs},i}$ represents the individual measurements of reflection i and its symmetry equivalent reflection.

‡PhP is the phasing power = $\sqrt{\sum F_{\text{H}}^2} / \sqrt{\sum (|F_{\text{PHobs}}| - |F_{\text{PHcalc}}|)^2}$, where F_{PH} and F_{H} are the derivative and calculated heavy-atom structure factors, respectively.

$\S R_{\text{cullis}} = \sum_i |F_{\text{PH}} \pm F_{\text{P}}| - F_{\text{Hcalc}} / \sum_i |F_{\text{PH}} \pm F_{\text{P}}|$, where F_{PH} , F_{P} and F_{H} are the derivative, native and calculated heavy-atom structure factors, respectively.

|| An anomalous signal to 4 Å resolution was used. The overall figure of merit for both derivatives, including the anomalous signal, was 0.37–2.9 Å.

$\P R = \sum_i |F_{\text{obs}} - F_{\text{calc}}| / \sum_i |F_{\text{obs}}|$ (on all data 1.9–20.0 Å).

$\# R_{\text{free}} = \sum_{\text{IKICT}} (|F_{\text{obs}}| - |F_{\text{calc}}|)^2 / \sum_{\text{IKICT}} |F_{\text{obs}}|^2$, where $\sum_{\text{IKICT}}$ are reflections belonging to a test set of 10% of the data.

r.m.s., root mean square.

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Correspondence and requests for materials should be addressed to N.C.J.S. (e-mail: natalie@byron.biochem.ubc.ca).

errata

Reconciling the spectrum of Sagittarius A* with a two-temperature plasma model

Rohan Mahadevan

Nature **394**, 651–653 (1998)

A misleading typographical error was introduced into the second sentence of the bold introductory paragraph of this Letter: the word “infrared” should be “inferred”. □

Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence

S. T. Cole, R. Brosch, J. Parkhill, T. Garnier, C. Churcher, D. Harris, S. V. Gordon, K. Eiglmeier, S. Gas, C. E. Barry III, F. Tekaia, K. Badcock, D. Basham, D. Brown, T. Chillingworth, R. Connor, R. Davies, K. Devlin, T. Feltwell, S. Gentles, N. Hamlin, S. Holroyd, T. Hornsby, K. Jagels, A. Krogh, J. McLean, S. Moule, L. Murphy, K. Oliver, J. Osborne, M. A. Quail, M.-A. Rajandream, J. Rogers, S. Rutter, K. Seeger, J. Skelton, R. Squares, S. Squares, J. E. Sulston, K. Taylor, S. Whitehead & B. G. Barrell

Nature **393**, 537–544 (1998)

As a result of an error during film output, Table 1 was published with some symbols missing. The correct version can be found at <http://www.sanger.ac.uk> and is reproduced again here (following pages).

Also, in Fig. 2, we incorrectly labelled Rv0649 as *fadD37* instead of *fabD2*. Two of the genes for mycolyl transferases were inverted: Rv0129c encodes antigen 85C and not 85C' as stated, whereas Rv3803c codes for the secreted protein MPT51 and not antigen 85C (*Infect. Immun.* **59**, 372–382; 1991); Rv3803c is now designated *fbpD*. We thank Morten Harboe and Harald Wiker for drawing this to our attention.

The sequence of Rv0746 from *M. bovis* BCG-Pasteur presented in Fig. 5b was incorrect and should have shown a 16-codon deletion instead of 29, as indicated here:

```
H37Rv . . . . . GSGAPGGAGGAAGLWGTGGAGGAGGSSAGGGGAGGAGGAGGWLGDGGAGGIGGAST . . .
. . . . . : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : :
BCG . . . . . GSGAPGGAGGAAGLWGTGGA-----GGAGGWLGDGGAGGIGGAST . . .
```

Table 1. Functional classification of *Mycobacterium tuberculosis* protein-coding genes

I. Small-molecule metabolism

A. Degradation

1. Carbon compounds

Rv0186	<i>bglS</i>	β-glucosidase
Rv2202c	<i>cbhK</i>	carbohydrate kinase
Rv0727c	<i>fucA</i>	L-fucose phosphate aldolase
Rv1731	<i>gabD1</i>	succinate-semialdehyde dehydrogenase
Rv0234c	<i>gabD2</i>	succinate-semialdehyde dehydrogenase
Rv0501	<i>galE1</i>	UDP-glucose 4-epimerase
Rv0536	<i>galE2</i>	UDP-glucose 4-epimerase
Rv0620	<i>galK</i>	galactokinase
Rv0619	<i>galT</i>	galactose-1-phosphate uridylyltransferase C-term
Rv0618	<i>galT'</i>	galactose-1-phosphate uridylyltransferase N-term
Rv0993	<i>galU</i>	UTP-glucose-1-phosphate uridylyltransferase
Rv3696c	<i>glpK</i>	ATP:glycerol 3-phosphotransferase
Rv3255c	<i>manA</i>	mannose-6-phosphate isomerase
Rv3441c	<i>mraA</i>	phosphoglucomutase or phosphomannomutase
Rv0118c	<i>oxcA</i>	oxalyl-CoA decarboxylase
Rv3068c	<i>pgmA</i>	phosphoglucomutase
Rv3257c	<i>pmmA</i>	phosphomannomutase
Rv3308	<i>pmmB</i>	phosphomannomutase
Rv2702	<i>ppgK</i>	polyphosphate glucokinase
Rv0408	<i>pta</i>	phosphate acetyltransferase
Rv0729	<i>xyfB</i>	xylulose kinase
Rv1096	-	carbohydrate degrading enzyme

2. Amino acids and amines

Rv1905c	<i>aoa</i>	D-amino acid oxidase
Rv2531c	<i>adi</i>	ornithine/arginine decarboxylase
Rv2780	<i>ald</i>	L-alanine dehydrogenase
Rv1538c	<i>ansA</i>	L-asparaginase
Rv1001	<i>arcA</i>	arginine deiminase
Rv0753c	<i>mmsA</i>	methylmalmonate semialdehyde dehydrogenase
Rv0751c	<i>mmsB</i>	methylmalmonate semialdehyde oxidoreductase
Rv1187	<i>rocA</i>	pyrroline-5-carboxylate dehydrogenase
Rv2322c	<i>rocD1</i>	ornithine aminotransferase
Rv2321c	<i>rocD2</i>	ornithine aminotransferase
Rv1848	<i>ureA</i>	urease γ subunit
Rv1849	<i>ureB</i>	urease β subunit
Rv1850	<i>ureC</i>	urease α subunit
Rv1853	<i>ureD</i>	urease accessory protein
Rv1851	<i>ureF</i>	urease accessory protein
Rv1852	<i>ureG</i>	urease accessory protein
Rv2913c	-	probable D-amino acid aminohydrolase
Rv3551	-	possible glutamate CoA-transferase

3. Fatty acids

Rv2501c	<i>accA1</i>	acetyl/propionyl-CoA carboxylase, α subunit
Rv0973c	<i>accA2</i>	acetyl/propionyl-CoA carboxylase, α subunit
Rv2502c	<i>accD1</i>	acetyl/propionyl-CoA carboxylase, β subunit
Rv0974c	<i>accD2</i>	acetyl/propionyl-CoA carboxylase, β subunit
Rv3667	<i>acs</i>	acetyl-CoA synthase
Rv3409c	<i>choD</i>	cholesterol oxidase
Rv0222	<i>echA1</i>	enoyl-CoA hydratase/isomerase superfamily
Rv0456c	<i>echA2</i>	enoyl-CoA hydratase/isomerase superfamily
Rv0632c	<i>echA3</i>	enoyl-CoA hydratase/isomerase superfamily
Rv0673	<i>echA4</i>	enoyl-CoA hydratase/isomerase superfamily
Rv0675	<i>echA5</i>	enoyl-CoA hydratase/isomerase superfamily
Rv0905	<i>echA6</i>	enoyl-CoA hydratase/isomerase superfamily (aka <i>echH</i>)
Rv0971c	<i>echA7</i>	enoyl-CoA hydratase/isomerase superfamily
Rv1070c	<i>echA8</i>	enoyl-CoA hydratase/isomerase superfamily
Rv1071c	<i>echA9</i>	enoyl-CoA hydratase/isomerase superfamily
Rv1142c	<i>echA10</i>	enoyl-CoA hydratase/isomerase superfamily
Rv1141c	<i>echA11</i>	enoyl-CoA hydratase/isomerase superfamily
Rv1472	<i>echA12</i>	enoyl-CoA hydratase/isomerase superfamily
Rv1935c	<i>echA13</i>	enoyl-CoA hydratase/isomerase superfamily
Rv2486	<i>echA14</i>	enoyl-CoA hydratase/isomerase superfamily
Rv2679	<i>echA15</i>	enoyl-CoA hydratase/isomerase superfamily

Rv2831	<i>echA16</i>	superfamily enoyl-CoA hydratase/isomerase
Rv3039c	<i>echA17</i>	superfamily enoyl-CoA hydratase/isomerase
Rv3373	<i>echA18</i>	superfamily enoyl-CoA hydratase/isomerase
Rv3374	<i>echA18'</i>	superfamily, N-term enoyl-CoA hydratase/isomerase
Rv3516	<i>echA19</i>	superfamily, C-term enoyl-CoA hydratase/isomerase
Rv3550	<i>echA20</i>	superfamily enoyl-CoA hydratase/isomerase
Rv3774	<i>echA21</i>	superfamily enoyl-CoA hydratase/isomerase
Rv0859	<i>fadA</i>	β oxidation complex, β subunit (acetyl-CoA C-acetyltransferase)
Rv0243	<i>fadA2</i>	acetyl-CoA C-acetyltransferase
Rv1074c	<i>fadA3</i>	acetyl-CoA C-acetyltransferase
Rv1323	<i>fadA4</i>	acetyl-CoA C-acetyltransferase (aka <i>thiL</i>)
Rv3546	<i>fadA5</i>	acetyl-CoA C-acetyltransferase
Rv3556c	<i>fadA6</i>	acetyl-CoA C-acetyltransferase
Rv0860	<i>fadB</i>	β oxidation complex, α subunit (multiple activities)
Rv0468	<i>fadB2</i>	3-hydroxyacyl-CoA dehydrogenase
Rv1715	<i>fadB3</i>	3-hydroxyacyl-CoA dehydrogenase
Rv3141	<i>fadB4</i>	3-hydroxyacyl-CoA dehydrogenase
Rv1912c	<i>fadB5</i>	3-hydroxyacyl-CoA dehydrogenase
Rv1750c	<i>fadD1</i>	acyl-CoA synthase
Rv0270	<i>fadD2</i>	acyl-CoA synthase
Rv3561	<i>fadD3</i>	acyl-CoA synthase
Rv0214	<i>fadD4</i>	acyl-CoA synthase
Rv0166	<i>fadD5</i>	acyl-CoA synthase
Rv1206	<i>fadD6</i>	acyl-CoA synthase
Rv0119	<i>fadD7</i>	acyl-CoA synthase
Rv0551c	<i>fadD8</i>	acyl-CoA synthase
Rv2590	<i>fadD9</i>	acyl-CoA synthase
Rv0099	<i>fadD10</i>	acyl-CoA synthase
Rv1550	<i>fadD11</i>	acyl-CoA synthase, N-term
Rv1549	<i>fadD11'</i>	acyl-CoA synthase, C-term
Rv1427c	<i>fadD12</i>	acyl-CoA synthase
Rv3089	<i>fadD13</i>	acyl-CoA synthase
Rv1058	<i>fadD14</i>	acyl-CoA synthase
Rv2187	<i>fadD15</i>	acyl-CoA synthase
Rv0852	<i>fadD16</i>	acyl-CoA synthase
Rv3506	<i>fadD17</i>	acyl-CoA synthase
Rv3513c	<i>fadD18</i>	acyl-CoA synthase
Rv3515c	<i>fadD19</i>	acyl-CoA synthase
Rv1185c	<i>fadD21</i>	acyl-CoA synthase
Rv2948c	<i>fadD22</i>	acyl-CoA synthase
Rv3826	<i>fadD23</i>	acyl-CoA synthase
Rv1529	<i>fadD24</i>	acyl-CoA synthase
Rv1521	<i>fadD25</i>	acyl-CoA synthase
Rv2930	<i>fadD26</i>	acyl-CoA synthase
Rv0275c	<i>fadD27</i>	acyl-CoA synthase
Rv2941	<i>fadD28</i>	acyl-CoA synthase
Rv2950c	<i>fadD29</i>	acyl-CoA synthase
Rv0404	<i>fadD30</i>	acyl-CoA synthase
Rv1925	<i>fadD31</i>	acyl-CoA synthase
Rv3801c	<i>fadD32</i>	acyl-CoA synthase
Rv1345	<i>fadD33</i>	acyl-CoA synthase
Rv0035	<i>fadD34</i>	acyl-CoA synthase
Rv2505c	<i>fadD35</i>	acyl-CoA synthase
Rv1193	<i>fadD36</i>	acyl-CoA synthase
Rv0131c	<i>fadE1</i>	acyl-CoA dehydrogenase
Rv0154c	<i>fadE2</i>	acyl-CoA dehydrogenase
Rv0215c	<i>fadE3</i>	acyl-CoA dehydrogenase
Rv0231	<i>fadE4</i>	acyl-CoA dehydrogenase
Rv0244c	<i>fadE5</i>	acyl-CoA dehydrogenase
Rv0271c	<i>fadE6</i>	acyl-CoA dehydrogenase
Rv0400c	<i>fadE7</i>	acyl-CoA dehydrogenase
Rv0672	<i>fadE8</i>	acyl-CoA dehydrogenase (aka <i>aidB</i>)
Rv0752c	<i>fadE9</i>	acyl-CoA dehydrogenase
Rv0873	<i>fadE10</i>	acyl-CoA dehydrogenase
Rv0972c	<i>fadE12</i>	acyl-CoA dehydrogenase
Rv0975c	<i>fadE13</i>	acyl-CoA dehydrogenase
Rv1346	<i>fadE14</i>	acyl-CoA dehydrogenase
Rv1467c	<i>fadE15</i>	acyl-CoA dehydrogenase
Rv1679	<i>fadE16</i>	acyl-CoA dehydrogenase
Rv1934c	<i>fadE17</i>	acyl-CoA dehydrogenase
Rv1933c	<i>fadE18</i>	acyl-CoA dehydrogenase
Rv2500c	<i>fadE19</i>	acyl-CoA dehydrogenase (aka <i>mmgC</i>)
Rv2724c	<i>fadE20</i>	acyl-CoA dehydrogenase
Rv2789c	<i>fadE21</i>	acyl-CoA dehydrogenase
Rv3061c	<i>fadE22</i>	acyl-CoA dehydrogenase
Rv3140	<i>fadE23</i>	acyl-CoA dehydrogenase
Rv3139	<i>fadE24</i>	acyl-CoA dehydrogenase
Rv3274c	<i>fadE25</i>	acyl-CoA dehydrogenase
Rv3504	<i>fadE26</i>	acyl-CoA dehydrogenase
Rv3505	<i>fadE27</i>	acyl-CoA dehydrogenase
Rv3544c	<i>fadE28</i>	acyl-CoA dehydrogenase

Rv3543c	<i>fadE29</i>	acyl-CoA dehydrogenase
Rv3560c	<i>fadE30</i>	acyl-CoA dehydrogenase
Rv3562	<i>fadE31</i>	acyl-CoA dehydrogenase
Rv3563	<i>fadE32</i>	acyl-CoA dehydrogenase
Rv3564	<i>fadE33</i>	acyl-CoA dehydrogenase
Rv3573c	<i>fadE34</i>	acyl-CoA dehydrogenase
Rv3797	<i>fadE35</i>	acyl-CoA dehydrogenase
Rv3761c	<i>fadE36</i>	acyl-CoA dehydrogenase
Rv1175c	<i>fadH</i>	2,4-Dienoyl-CoA Reductase
Rv0855	<i>far</i>	fatty acyl-CoA racemase
Rv1143	<i>mcr</i>	α-methyl acyl-CoA racemase
Rv1492	<i>mutA</i>	methylmalonyl-CoA mutase, β subunit
Rv1493	<i>mutB</i>	methylmalonyl-CoA mutase, α subunit
Rv2504c	<i>scoA</i>	3-oxo acid:CoA transferase, α subunit
Rv2503c	<i>scoB</i>	3-oxo acid:CoA transferase, β subunit
Rv1136	-	probable carnitine racemase
Rv1683	-	possible acyl-CoA synthase

4. Phosphorous compounds

Rv2368c	<i>phoH</i>	ATP-binding <i>pho</i> regulon component
Rv1095	<i>phoH2</i>	PhoH-like protein
Rv3628	<i>ppa</i>	probable inorganic pyrophosphatase
Rv2984	<i>ppk</i>	polyphosphate kinase

B. Energy metabolism

1. Glycolysis

Rv1023	<i>eno</i>	enolase
Rv0363c	<i>fba</i>	fructose bisphosphate aldolase
Rv1436	<i>gap</i>	glyceraldehyde 3-phosphate dehydrogenase
Rv0489	<i>gpm</i>	phosphoglycerate mutase I
Rv3010c	<i>pfkA</i>	phosphofructokinase I
Rv2029c	<i>pfkB</i>	phosphofructokinase II
Rv0946c	<i>pgi</i>	glucose-6-phosphate isomerase
Rv1437	<i>pgk</i>	phosphoglycerate kinase
Rv1617	<i>pykA</i>	pyruvate kinase
Rv1438	<i>tpi</i>	triosephosphate isomerase
Rv2419c	-	putative phosphoglycerate mutase
Rv3837c	-	putative phosphoglycerate mutase

2. Pyruvate dehydrogenase

Rv2241	<i>aceE</i>	pyruvate dehydrogenase E1 component
Rv3303c	<i>lpdA</i>	dihydrolipoamide dehydrogenase
Rv2497c	<i>pdhA</i>	pyruvate dehydrogenase E1 component α subunit
Rv2496c	<i>pdhB</i>	pyruvate dehydrogenase E1 component β subunit
Rv2495c	<i>pdhC</i>	dihydrolipoamide acetyltransferase
Rv0462	-	probable dihydrolipoamide dehydrogenase

3. TCA cycle

Rv1475c	<i>acon</i>	aconitate hydratase
Rv0889c	<i>citA</i>	citrate synthase 2
Rv2498c	<i>citE</i>	citrate lyase β chain
Rv1098c	<i>fum</i>	fumarase
Rv1131	<i>glfA1</i>	citrate synthase 3
Rv0896	<i>glfA2</i>	citrate synthase 1
Rv3339c	<i>icd1</i>	isocitrate dehydrogenase
Rv0066c	<i>icd2</i>	isocitrate dehydrogenase
Rv0794c	<i>lpdB</i>	dihydrolipoamide dehydrogenase
Rv1240	<i>mdh</i>	malate dehydrogenase
Rv2967c	<i>pca</i>	pyruvate carboxylase
Rv3318	<i>sdhA</i>	succinate dehydrogenase A
Rv3319	<i>sdhB</i>	succinate dehydrogenase B
Rv3316	<i>sdhC</i>	succinate dehydrogenase C subunit
Rv3317	<i>sdhD</i>	succinate dehydrogenase D subunit
Rv1248c	<i>sucA</i>	2-oxoglutarate dehydrogenase
Rv2215	<i>sucB</i>	dihydrolipoamide succinyltransferase
Rv0951	<i>sucC</i>	succinyl-CoA synthase β chain
Rv0952	<i>sucD</i>	succinyl-CoA synthase α chain

4. Glyoxylate bypass

Rv0467	<i>aceA</i>	isocitrate lyase
Rv1915	<i>aceAa</i>	isocitrate lyase, α module
Rv1916	<i>aceAb</i>	isocitrate lyase, β module
Rv1837c	<i>glcB</i>	malate synthase
Rv3323c	<i>gphA</i>	phosphoglycolate phosphatase

5. Pentose phosphate pathway

Rv1445c	<i>devB</i>	glucose-6-phosphate 1-dehydrogenase
Rv1844c	<i>gnd</i>	6-phosphogluconate dehydrogenase (Gram -)
Rv1122	<i>gnd2</i>	6-phosphogluconate dehydrogenase (Gram +)
Rv1446c	<i>opcA</i>	unknown function, may aid G6PDH

Rv2436 *rbsK* ribokinase
 Rv1408 *rpe* ribulose-phosphate 3-epimerase
 Rv2465c *rpi* phosphopentose isomerase
 Rv1448c *tal* transaldolase
 Rv1449c *tkl* transketolase
 Rv1121 *zwf* glucose-6-phosphate 1-dehydrogenase
 Rv1447c *zwf2* glucose-6-phosphate 1-dehydrogenase

6. Respiration

a. aerobic
 Rv0527 *ccsA* cytochrome *c*-type biogenesis protein
 Rv0529 *ccsB* cytochrome *c*-type biogenesis protein
 Rv1451 *ctaB* cytochrome *c* oxidase assembly factor
 Rv2200c *ctaC* cytochrome *c* oxidase chain II
 Rv3043c *ctaD* cytochrome *c* oxidase polypeptide I
 Rv2193 *ctaE* cytochrome *c* oxidase polypeptide III
 Rv1542c *glbN* hemoglobin-like, oxygen carrier
 Rv2470 *glbO* hemoglobin-like, oxygen carrier
 Rv2249c *glpD1* glycerol-3-phosphate dehydrogenase
 Rv3302c *glpD2* glycerol-3-phosphate dehydrogenase
 Rv0694 *lldD1* L-lactate dehydrogenase (cytochrome)
 Rv1872c *lldD2* L-lactate dehydrogenase
 Rv1854c *ndh* probable NADH dehydrogenase
 Rv3145 *nuoA* NADH dehydrogenase chain A
 Rv3146 *nuoB* NADH dehydrogenase chain B
 Rv3147 *nuoC* NADH dehydrogenase chain C
 Rv3148 *nuoD* NADH dehydrogenase chain D
 Rv3149 *nuoE* NADH dehydrogenase chain E
 Rv3150 *nuoF* NADH dehydrogenase chain F
 Rv3151 *nuoG* NADH dehydrogenase chain G
 Rv3152 *nuoH* NADH dehydrogenase chain H
 Rv3153 *nuoI* NADH dehydrogenase chain I
 Rv3154 *nuoJ* NADH dehydrogenase chain J
 Rv3155 *nuoK* NADH dehydrogenase chain K
 Rv3156 *nuoL* NADH dehydrogenase chain L
 Rv3157 *nuoM* NADH dehydrogenase chain M
 Rv3158 *nuoN* NADH dehydrogenase chain N
 Rv2195 *qcrA* Rieske iron-sulphur component of *ubiQ*-cytB reductase
 Rv2196 *qcrB* cytochrome β component of *ubiQ*-cytB reductase
 Rv2194 *qcrC* cytochrome *b/c* component of *ubiQ*-cytB reductase

b. anaerobic

Rv2392 *cysH* 3'-phosphoadenylylsulfate (PAPS) reductase
 Rv2899c *fdhD* affects formate dehydrogenase-N molybdopter-in-containing oxidoreductase
 Rv2900c *fdhF* affects formate dehydrogenase-N molybdopter-in-containing oxidoreductase
 Rv1552 *frdA* fumarate reductase flavoprotein subunit
 Rv1553 *frdB* fumarate reductase iron sulphur protein
 Rv1554 *frdC* fumarate reductase 15kD anchor protein
 Rv1555 *frdD* fumarate reductase 13kD anchor protein
 Rv1161 *narG* nitrate reductase α subunit
 Rv1162 *narH* nitrate reductase β chain
 Rv1164 *narI* nitrate reductase γ chain
 Rv1163 *narJ* nitrate reductase δ chain
 Rv1736c *narX* fused nitrate reductase
 Rv2391 *nirA* probable nitrite reductase/sulphite reductase
 Rv0252 *nirB* nitrite reductase flavoprotein
 Rv0253 *nirD* probable nitrite reductase small subunit

c. Electron transport

Rv0409 *ackA* acetate kinase
 Rv1623c *appC* cytochrome *bd-II* oxidase subunit I
 Rv1622c *cydB* cytochrome *d* ubiquinol oxidase subunit II
 Rv1620c *cydC* ABC transporter
 Rv1621c *cydD* ABC transporter
 Rv2007c *fdxA* ferredoxin
 Rv3554 *fdxB* ferredoxin
 Rv1177 *fdxC* ferredoxin 4Fe-4S
 Rv3503c *fdxD* probable ferredoxin
 Rv3029c *fixA* electron transfer flavoprotein β subunit
 Rv3028c *fixB* electron transfer flavoprotein α subunit
 Rv3106 *fprA* adrenodoxin and NADPH ferredoxin reductase
 Rv0886 *fprB* ferredoxin, ferredoxin-NADP reductase
 Rv3251c *rubA* rubredoxin A

Rv3250c *rubB* rubredoxin B

7. Miscellaneous oxidoreductases and oxygenases 171

8. ATP-proton motive force

Rv1308 *atpA* ATP synthase α chain
 Rv1304 *atpB* ATP synthase α chain
 Rv1311 *atpC* ATP synthase ϵ chain
 Rv1310 *atpD* ATP synthase β chain
 Rv1305 *atpE* ATP synthase γ chain
 Rv1306 *atpF* ATP synthase δ chain
 Rv1309 *atpG* ATP synthase γ chain
 Rv1307 *atpH* ATP synthase δ chain

C. Central intermediary metabolism

1. General

Rv2589 *gabT* 4-aminobutyrate aminotransferase
 Rv3432c *gadB* glutamate decarboxylase
 Rv1832 *gcvB* glycine decarboxylase
 Rv1826 *gcvH* glycine cleavage system H protein
 Rv2211c *gcvT* T protein of glycine cleavage system

Rv1213 *glgC* glucose-1-phosphate adenylyltransferase

Rv3842c *glpQ1* glycerophosphoryl diester phosphodiesterase

Rv0317c *glpQ2* glycerophosphoryl diester phosphodiesterase

Rv3566c *nhoA* N-hydroxyarylamino α -acetyltransferase

Rv0155 *pntAA* pyridine transhydrogenase subunit α 1

Rv0156 *pntAB* pyridine transhydrogenase subunit α 2

Rv0157 *pntB* pyridine transhydrogenase subunit β

Rv1127c *ppdK* similar to pyruvate, phosphate dikinase

2. Gluconeogenesis

Rv0211 *pckA* phosphoenolpyruvate carboxykinase
 Rv0069c *sdaA* L-serine dehydratase 1

3. Sugar nucleotides

Rv1512 *epiA* nucleotide sugar epimerase
 Rv3784 *epiB* probable UDP-galactose 4-epimerase
 Rv1511 *gmdA* GDP-mannose 4,6 dehydratase
 Rv0334 *rmlA* glucose-1-phosphate thymidyltransferase

Rv3264c *rmlA2* glucose-1-phosphate thymidyltransferase

Rv3464 *rmlB* dTDP-glucose 4,6-dehydratase

Rv3634c *rmlB2* dTDP-glucose 4,6-dehydratase

Rv3468c *rmlB3* dTDP-glucose 4,6-dehydratase

Rv3465 *rmlC* dTDP-4-dehydroxymannose 3,5-epimerase

Rv3266c *rmlD* dTDP-4-dehydroxymannose reductase

Rv0322 *udgA* UDP-glucose dehydrogenase/GDP-mannose 6-dehydrogenase

Rv3265c *wbbL* dTDP-rhamnosyl transferase

Rv1525 *wbbL2* dTDP-rhamnosyl transferase

Rv3400 - probable β -phosphoglucomutase

4. Amino sugars

Rv3436c *glmS* glucosamine-fructose-6-phosphate aminotransferase

5. Sulphur metabolism

Rv0711 *atsA* arylsulfatase
 Rv3299c *atsB* probable arylsulfatase
 Rv0663 *atsD* probable arylsulfatase
 Rv3077 *atsF* probable arylsulfatase
 Rv0296c *atsG* probable arylsulfatase
 Rv3796 *atsH* probable arylsulfatase
 Rv1285 *cysD* ATP:sulphurylase subunit 2
 Rv1286 *cysN* ATP:sulphurylase subunit 1
 Rv2131c *cysQ* homologue of *M.leprae* *cysQ*
 Rv3248c *sahH* adenosylhomocysteinase
 Rv3283 *sseA* thiosulfate sulfurtransferase
 Rv2291 *sseB* thiosulfate sulfurtransferase
 Rv3118 *sseC* thiosulfate sulfurtransferase
 Rv0814c *sseC2* thiosulfate sulfurtransferase
 Rv3762c - probable alkyl sulfatase

D. Amino acid biosynthesis

1. Glutamate family

Rv1654 *argB* acetylglutamate kinase
 Rv1652 *argC* N-acetyl- γ -glutamyl-phosphate reductase
 Rv1655 *argD* acetylornithine aminotransferase
 Rv1656 *argF* ornithine carbamoyltransferase
 Rv1658 *argG* arginosuccinate synthase
 Rv1659 *argH* arginosuccinate lyase
 Rv1653 *argJ* glutamate N-acetyltransferase
 Rv2220 *glnA1* glutamine synthase class I
 Rv2222c *glnA2* glutamine synthase class II

Rv1878 *glnA3* probable glutamine synthase
 Rv2860c *glnA4* probable glutamine synthase
 Rv2918c *glnD* uridylyltransferase
 Rv2221c *glnE* glutamate-ammonia-ligase
 Rv3859c *gltB* adenylyltransferase
 Rv3858c *gltD* ferredoxin-dependent glutamate synthase
 Rv3704c *gshA* small subunit of NADH-dependent glutamate synthase
 Rv2427c *proA* possible γ -glutamylcysteine synthase
 Rv2439c *proB* γ -glutamyl phosphate reductase
 Rv0500 *proC* glutamate 5-kinase
 pyrroline-5-carboxylate reductase

2. Aspartate family

Rv3708c *asd* aspartate semialdehyde dehydrogenase
 Rv3709c *ask* aspartokinase
 Rv2201 *asnB* asparagine synthase B
 Rv3565 *aspB* aspartate aminotransferase
 Rv0337c *aspC* aspartate aminotransferase
 Rv2753c *dapA* dihydrodipicolinate synthase
 Rv2773c *dapB* dihydrodipicolinate reductase
 Rv1202 *dapE* succinyl-diaminopimelate desuccinylase
 Rv2141c *dapE2* ArgE/DapE/Acy1/Cpg2/yscS family
 Rv2726c *dapF* diaminopimelate epimerase
 Rv1293 *lysA* diaminopimelate decarboxylase
 Rv3341 *metA* homoserine α -acetyltransferase
 Rv1079 *metB* cystathionine γ -synthase
 Rv3340 *metC* cystathionine β -lyase
 Rv1133c *metE* 5-methyltetrahydropteroylglutamate-homocysteine methyltransferase
 Rv2124c *metH* 5-methyltetrahydrofolate-homocysteine methyltransferase
 Rv1392 *metK* S-adenosylmethionine synthase
 Rv0391 *metZ* α -succinylhomoserine sulphydrylase
 Rv1294 *thrA* homoserine dehydrogenase
 Rv1296 *thrB* homoserine kinase
 Rv1295 *thrC* homoserine synthase

3. Serine family

Rv0815c *cysA2* thiosulfate sulfurtransferase
 Rv3117 *cysA3* thiosulfate sulfurtransferase
 Rv2335 *cysE* serine acetyltransferase
 Rv0511 *cysG* uroporphyrin-III α -methyltransferase
 Rv2847c *cysG2* multifunctional enzyme, siroheme synthase
 Rv2334 *cysK* cysteine synthase A
 Rv1336 *cysM* cysteine synthase B
 Rv1077 *cysM2* cystathionine β -synthase
 Rv0848 *cysM3* putative cysteine synthase
 Rv1093 *glyA* serine hydroxymethyltransferase
 Rv0070c *glyA2* serine hydroxymethyltransferase
 Rv2996c *serA* D-3-phosphoglycerate dehydrogenase
 Rv0505c *serB* probable phosphoserine phosphatase
 Rv3042c *serB2* C-term similar to phosphoserine phosphatase
 Rv0884c *serC* phosphoserine aminotransferase

4. Aromatic amino acid family

Rv3227 *aroA* 3-phosphoshikimate 1-carboxyvinyl transferase
 Rv2538c *aroB* 3-dehydroquinate synthase
 Rv2537c *aroD* 3-dehydroquinate dehydratase
 Rv2552c *aroE* shikimate 5-dehydrogenase
 Rv2540c *aroF* chorismate synthase
 Rv2178c *aroG* DAHP synthase
 Rv2539c *aroK* shikimate kinase I
 Rv3838c *pheA* prephenate dehydratase
 Rv1613 *trpA* tryptophan synthase α chain
 Rv1612 *trpB* tryptophan synthase β chain
 Rv1611 *trpC* indole-3-glycerol phosphate synthase
 Rv2192c *trpD* anthranilate phosphoribosyltransferase
 Rv1609 *trpE* anthranilate synthase component I
 Rv2386c *trpE2* anthranilate synthase component I
 Rv3754 *tyrA* prephenate dehydrogenase

5. Histidine

Rv1603 *hisA* phosphoribosylformimino-5-aminoimidazole carboxamide ribonucleotide isomerase
 Rv1601 *hisB* imidazole glycerol-phosphate dehydratase
 Rv1600 *hisC* histidinol-phosphate aminotransferase
 Rv3772 *hisC2* histidinol-phosphate aminotransferase
 Rv1599 *hisD* histidinol dehydrogenase

6. Pyruvate family		Rv3752c	-	probable cytidine/deoxycytidylate deaminase	Rv2338c	<i>moeW</i>	molybdopterin biosynthesis	
Rv3423c	<i>alr</i>	alanine racemase			Rv1681	<i>moeX</i>	weak similarity to <i>E. coli</i> MoeA	
					Rv1355c	<i>moeY</i>	weak similarity to <i>E. coli</i> MoeB	
					Rv3206c	<i>moeZ</i>	probably involved in molybdopterin biosynthesis	
7. Branched amino acid family		4. Salvage of nucleosides and nucleotides						
Rv1559	<i>ilvA</i>	threonine deaminase	Rv3313c	<i>add</i>	probable adenosine deaminase			
Rv3003c	<i>ilvB</i>	acetoactate synthase I large sub-unit	Rv2584c	<i>apt</i>	adenine phosphoribosyltransferases	Rv0865	<i>mog</i>	molybdopterin biosynthesis
Rv3470c	<i>ilvB2</i>	acetoactate synthase large sub-unit	Rv3315c	<i>cdd</i>	probable cytidine deaminase	5. Pantothenate		
			Rv3314c	<i>deoA</i>	thymidine phosphorylase	Rv1092c	<i>coaA</i>	pantothenate kinase
Rv3001c	<i>ilvC</i>	ketol-acid reductoisomerase	Rv0478	<i>deoC</i>	deoxyribose-phosphate aldolase	Rv2225	<i>panB</i>	3-methyl-2-oxobutanoate hydroxymethyltransferase
Rv0189c	<i>ilvD</i>	dihydroxy-acid dehydratase	Rv3307	<i>deoD</i>	probable purine nucleoside phosphorylase	Rv3602c	<i>panC</i>	pantoate-β-alanine ligase
Rv2210c	<i>ilvE</i>	branched-chain-amino-acid transaminase	Rv3624c	<i>hpt</i>	probable hypoxanthine-guanine phosphoribosyltransferase	Rv3601c	<i>panD</i>	aspartate 1-decarboxylase
Rv1820	<i>ilvG</i>	acetoactate synthase II	Rv3393	<i>iunH</i>	probable inosine-uridine preferring nucleoside hydrolase	6. Pyridoxine		
Rv3002c	<i>ilvN</i>	acetoactate synthase I small sub-unit	Rv0535	<i>pnp</i>	phosphorylase from Pnp/MtaP family 2	Rv2607	<i>pdxH</i>	pyridoxamine 5'-phosphate oxidase
Rv3509c	<i>ilvX</i>	probable acetohydroxyacid synthase I large subunit	Rv3309c	<i>upp</i>	uracil phosphoribosyltransferase			
Rv3710	<i>leuA</i>	α-isopropyl malate synthase				7. Pyridine nucleotide		
Rv2995c	<i>leuB</i>	3-isopropylmalate dehydrogenase	5. Miscellaneous nucleoside/nucleotide reactions			Rv1594	<i>nadA</i>	quinolinate synthase
Rv2988c	<i>leuC</i>	3-isopropylmalate dehydratase large subunit	Rv0733	<i>adk</i>	probable adenylate kinase	Rv1595	<i>nadB</i>	L-aspartate oxidase
			Rv2364c	<i>bex</i>	GTP-binding protein of Era/ThdF family	Rv1596	<i>nadC</i>	nicotinate-nucleotide pyrophosphate
Rv2987c	<i>leuD</i>	3-isopropylmalate dehydratase small subunit				Rv0423c	<i>thiC</i>	thiamine synthesis, pyrimidine moiety
			Rv1712	<i>cmk</i>	cytidylate kinase			
			Rv2344c	<i>dgt</i>	probable deoxyguanosine triphosphate hydrolase			
<i>E. Polyamine synthesis</i>								
Rv2601	<i>speE</i>	spermidine synthase	Rv2404c	<i>lepA</i>	GTP-binding protein LepA	8. Thiamine		
			Rv2727c	<i>miaA</i>	tRNA 8(2)-isopentenylpyrophosphate transferase	Rv0422c	<i>thiD</i>	phosphomethylpyrimidine kinase
<i>F. Purines, pyrimidines, nucleosides and nucleotides</i>						Rv0414c	<i>thiE</i>	thiamine synthesis, thiazole moiety
1. Purine ribonucleotide biosynthesis								
Rv1389	<i>gmk</i>	putative guanylate kinase	Rv2445c	<i>ndkA</i>	nucleoside diphosphate kinase	Rv0417	<i>thiG</i>	thiamine synthesis, thiazole moiety
Rv3396c	<i>guaA</i>	GMP synthase	Rv2440c	<i>obg</i>	Obg GTP-binding protein	Rv2977c	<i>thiL</i>	probable thiamine-monophosphate kinase
Rv1843c	<i>guaB1</i>	inosine-5'-monophosphate dehydrogenase	Rv2583c	<i>relA</i>	(p)ppGpp synthase I			
Rv3411c	<i>guaB2</i>	inosine-5'-monophosphate dehydrogenase				9. Riboflavin		
Rv3410c	<i>guaB3</i>	inosine-5'-monophosphate dehydrogenase				Rv1940	<i>ribA</i>	GTP cyclohydrolase II
Rv1017c	<i>prsA</i>	ribose-phosphate pyrophosphokinase	Rv1589	<i>bioB</i>	adenosylmethionine-8-amino-7-oxononanoate aminotransferase	Rv1415	<i>ribA2</i>	probable GTP cyclohydrolase II
Rv0357c	<i>purA</i>	adenylosuccinate synthase	Rv1570	<i>bioD</i>	biotin synthase	Rv1412	<i>ribC</i>	riboflavin synthase α chain
Rv0777	<i>purB</i>	adenylosuccinate lyase	Rv1569	<i>bioF</i>	dethiobiotin synthase	Rv2671	<i>ribD</i>	probable riboflavin deaminase
Rv0780	<i>purC</i>	phosphoribosylaminoimidazole-succinocarboxamide synthase				Rv2786c	<i>ribF</i>	riboflavin kinase
			Rv0032	<i>bioF2</i>	8-amino-7-oxononanoate synthase	Rv1409	<i>ribG</i>	riboflavin biosynthesis
Rv0772	<i>purD</i>	phosphoribosylamine-glycine ligase				Rv1416	<i>ribH</i>	riboflavin synthase β chain
Rv3275c	<i>purE</i>	phosphoribosylaminoimidazole carboxylase	Rv3279c	<i>birA</i>	C-terminal similar to <i>B. subtilis</i> BioF	Rv3300c	-	probable deaminase, riboflavin synthesis
Rv0808	<i>purF</i>	amidophosphoribosyltransferase	Rv1442	<i>bisC</i>	biotin apo-protein ligase			
Rv0957	<i>purH</i>	phosphoribosylaminoimidazole-carboxamide formyltransferase	Rv0089	-	biotin sulfoxide reductase	10. Thioredoxin, glutaredoxin and mycothiol		
Rv3276c	<i>purK</i>	phosphoribosylaminoimidazole carboxylase ATPase subunit			possible <i>bioC</i> biotin synthesis gene	Rv0773c	<i>ggtA</i>	putative γ-glutamyl transpeptidase
Rv0803	<i>purL</i>	phosphoribosylformylglycinamide synthase II	2. Folic acid			Rv2394	<i>ggtB</i>	γ-glutamyltranspeptidase precursor
Rv0809	<i>purM</i>	5'-phosphoribosyl-5-aminoimidazole synthase	Rv2763c	<i>dfra</i>	dihydrofolate reductase	Rv2855	<i>gorA</i>	glutathione reductase homologue
Rv0956	<i>purN</i>	phosphoribosylglycinamide formyltransferase I	Rv2447c	<i>foIC</i>	folypolyglutamate synthase	Rv0816c	<i>thiX</i>	equivalent to <i>M. leprae</i> ThiX
Rv0788	<i>purQ</i>	phosphoribosylformylglycinamide synthase I	Rv3356c	<i>foID</i>	methylene tetrahydrofolate dehydrogenase	Rv1470	<i>trxA</i>	thioredoxin
Rv0389	<i>purT</i>	phosphoribosylglycinamide formyltransferase II				Rv1471	<i>trxB</i>	thioredoxin reductase
Rv2964	<i>purU</i>	formyltetrahydrofolate deformylase	Rv3609c	<i>foIE</i>	GTP cyclohydrolase I	Rv3913	<i>trxB2</i>	thioredoxin reductase
			Rv3606c	<i>foIK</i>	7,8-dihydro-6-hydroxymethylpterin pyrophosphokinase	Rv3914	<i>trxC</i>	thioredoxin
			Rv3608c	<i>foIP</i>	dihydropterolate synthase	11. Menaquinone, PQQ, ubiquinone and other		
			Rv1207	<i>foIP2</i>	dihydropterolate synthase	Rv2682c	<i>dxs</i>	1-deoxy-D-xylulose 5-phosphate synthase
			Rv3607c	<i>foIX</i>	may be involved in folate biosynthesis			
						Rv0562	<i>grcC1</i>	heptaprenyl diphosphate synthase II
			Rv0013	<i>pabA</i>	p-aminobenzoate synthase glutamine amidotransferase	Rv0989c	<i>grcC2</i>	heptaprenyl diphosphate synthase II
			Rv1005c	<i>pabB</i>	p-aminobenzoate synthase			
			Rv0812	<i>pabC</i>	aminodeoxychorismate lyase	Rv3398c	<i>idsA</i>	geranylgeranyl pyrophosphate synthase
						Rv2173	<i>idsA2</i>	geranylgeranyl pyrophosphate synthase
2. Pyrimidine ribonucleotide biosynthesis		3. Lipoate				Rv3383c	<i>idsB</i>	transfergeranyl, similar geranyl pyrophosphate synthase
Rv1383	<i>carA</i>	carbamoyl-phosphate synthase subunit	Rv2218	<i>lipA</i>	lipoate biosynthesis protein A			
Rv1384	<i>carB</i>	carbamoyl-phosphate synthase subunit	Rv2217	<i>lipB</i>	lipoate biosynthesis protein B	Rv0534c	<i>menA</i>	4-dihydroxy-2-naphthoate octaprenyltransferase
Rv1380	<i>pyrB</i>	aspartate carbamoyltransferase	Rv3109	<i>moaA</i>	molybdenum cofactor biosynthesis, protein A	Rv0548c	<i>menB</i>	naphthoate synthase
Rv1381	<i>pyrC</i>	dihydroorotate				Rv0553	<i>menC</i>	o-succinylbenzoate-CoA synthase
Rv2139	<i>pyrD</i>	dihydroorotate dehydrogenase	Rv0869c	<i>moaA2</i>	molybdenum cofactor biosynthesis, protein A	Rv0555	<i>menD</i>	2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate synthase
Rv1385	<i>pyrF</i>	orotidine 5'-phosphate decarboxylase						
			Rv0438c	<i>moaA3</i>	molybdenum cofactor biosynthesis, protein A	Rv0542c	<i>menE</i>	o-succinylbenzoic acid-CoA ligase
Rv1699	<i>pyrG</i>	CTP synthase				Rv3853	<i>menG</i>	S-adenosylmethionine: 2-demethylmenaquinone
Rv2883c	<i>pyrH</i>	uridyate kinase	Rv3110	<i>moaB</i>	molybdenum cofactor biosynthesis, protein B			
Rv0382c	<i>umpA</i>	probable uridine 5'-monophosphate synthase				Rv3397c	<i>phyA</i>	phytoene synthase
			Rv0984	<i>moaB2</i>	molybdenum cofactor biosynthesis, protein B	Rv0693	<i>pqqE</i>	coenzyme PQQ synthesis
			Rv3111	<i>moaC</i>	molybdenum cofactor biosynthesis, protein C	Rv0558	<i>ubiE</i>	ubiquinone/menaquinone biosynthesis methyltransferase
3. 2'-deoxyribonucleotide metabolism								
Rv0321	<i>dcd</i>	deoxycytidine triphosphate deaminase	Rv0864	<i>moaC2</i>	molybdenum cofactor biosynthesis, protein C			
Rv2697c	<i>dut</i>	deoxyuridine triphosphatase				12. Heme and porphyrin		
Rv0233	<i>nrdB</i>	ribonucleoside-diphosphate reductase B2 (eukaryotic-like)	Rv3324c	<i>moaC3</i>	molybdenum cofactor biosynthesis, protein C	Rv0509	<i>hemA</i>	glutamyl-tRNA reductase
						Rv0512	<i>hemB</i>	δ-aminolevulinic acid dehydratase
Rv3051c	<i>nrdE</i>	ribonucleoside diphosphate reductase α chain	Rv3112	<i>moaD</i>	molybdopterin converting factor subunit 1	Rv0510	<i>hemC</i>	porphobilinogen deaminase

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Rv0823c	-	family) transcriptional regulator (NifR3/Smm1 family)	Rv3160c	-	putative transcriptional regulator	Rv0018c	<i>ppp</i>	truncated putative phosphoprotein phosphatase
Rv0827c	-	transcriptional regulator (ArsR family)	Rv3167c	-	putative transcriptional regulator	Rv2234	<i>ptpA</i>	low molecular weight protein-tyrosine-phosphatase
Rv0890c	-	transcriptional regulator (LuxR/UhpA family)	Rv3173c	-	transcriptional regulator (TetR/AcrR family)	Rv0153c	-	putative protein-tyrosine-phosphatase
Rv0891c	-	putative transcriptional regulator	Rv3183	-	putative transcriptional regulator	II. Macromolecule metabolism <i>A. Synthesis and modification of macromolecules</i> 1. Ribosomal protein synthesis and modification Rv3420c <i>rimI</i> ribosomal protein S18 acetyl transferase Rv0995 <i>rimJ</i> acetylation of 30S S5 subunit Rv0641 <i>rplA</i> 50S ribosomal protein L1 Rv0704 <i>rplB</i> 50S ribosomal protein L2 Rv0701 <i>rplC</i> 50S ribosomal protein L3 Rv0702 <i>rplD</i> 50S ribosomal protein L4 Rv0716 <i>rplE</i> 50S ribosomal protein L5 Rv0719 <i>rplF</i> 50S ribosomal protein L6 Rv0056 <i>rplI</i> 50S ribosomal protein L9 Rv0651 <i>rplJ</i> 50S ribosomal protein L10 Rv0640 <i>rplK</i> 50S ribosomal protein L11 Rv0652 <i>rplL</i> 50S ribosomal protein L7/L12 Rv3443c <i>rplM</i> 50S ribosomal protein L13 Rv0714 <i>rplN</i> 50S ribosomal protein L14 Rv0723 <i>rplO</i> 50S ribosomal protein L15 Rv0708 <i>rplP</i> 50S ribosomal protein L16 Rv3456c <i>rplQ</i> 50S ribosomal protein L17 Rv0720 <i>rplR</i> 50S ribosomal protein L18 Rv2904c <i>rplS</i> 50S ribosomal protein L19 Rv1643 <i>rplT</i> 50S ribosomal protein L20 Rv2442c <i>rplU</i> 50S ribosomal protein L21 Rv0706 <i>rplV</i> 50S ribosomal protein L22 Rv0703 <i>rplW</i> 50S ribosomal protein L23 Rv0715 <i>rplX</i> 50S ribosomal protein L24 Rv1015c <i>rplY</i> 50S ribosomal protein L25 Rv2441c <i>rpmA</i> 50S ribosomal protein L27 Rv1010c <i>rpmB</i> 50S ribosomal protein L28 Rv2058c <i>rpmB2</i> 50S ribosomal protein L28 Rv0709 <i>rpmC</i> 50S ribosomal protein L29 Rv0722 <i>rpmD</i> 50S ribosomal protein L30 Rv1298 <i>rpmE</i> 50S ribosomal protein L31 Rv2057c <i>rpmG</i> 50S ribosomal protein L33 Rv3924c <i>rpmH</i> 50S ribosomal protein L34 Rv1642 <i>rpmI</i> 50S ribosomal protein L35 Rv3461c <i>rpmJ</i> 50S ribosomal protein L36 Rv1630 <i>rpsA</i> 30S ribosomal protein S1 Rv2890c <i>rpsB</i> 30S ribosomal protein S2 Rv0707 <i>rpsC</i> 30S ribosomal protein S3 Rv3458c <i>rpsD</i> 30S ribosomal protein S4 Rv0721 <i>rpsE</i> 30S ribosomal protein S5 Rv0053 <i>rpsF</i> 30S ribosomal protein S6 Rv0683 <i>rpsG</i> 30S ribosomal protein S7 Rv0718 <i>rpsH</i> 30S ribosomal protein S8 Rv3442c <i>rpsI</i> 30S ribosomal protein S9 Rv0700 <i>rpsJ</i> 30S ribosomal protein S10 Rv3459c <i>rpsK</i> 30S ribosomal protein S11 Rv0682 <i>rpsL</i> 30S ribosomal protein S12 Rv3460c <i>rpsM</i> 30S ribosomal protein S13 Rv0717 <i>rpsN</i> 30S ribosomal protein S14 Rv2056c <i>rpsN2</i> 30S ribosomal protein S14 Rv2785c <i>rpsO</i> 30S ribosomal protein S15 Rv2909c <i>rpsP</i> 30S ribosomal protein S16 Rv0710 <i>rpsQ</i> 30S ribosomal protein S17 Rv0055 <i>rpsR</i> 30S ribosomal protein S18 Rv2055c <i>rpsR2</i> 30S ribosomal protein S18 Rv0705 <i>rpsS</i> 30S ribosomal protein S19 Rv2412 <i>rpsT</i> 30S ribosomal protein S20 Rv3241c - member of S30AE ribosomal protein family		
Rv0894	-	putative transcriptional regulator	Rv3208	-	transcriptional regulator (TetR/AcrR family)			
Rv1019	-	transcriptional regulator (TetR/AcrR family)	Rv3249c	-	transcriptional regulator (TetR/AcrR family)			
Rv1049	-	transcriptional regulator (MarR family)	Rv3291c	-	transcriptional regulator (Lrp/AsnC family)			
Rv1129c	-	transcriptional regulator (PbsX/Xre family)	Rv3295	-	transcriptional regulator (TetR/AcrR family)			
Rv1151c	-	putative transcriptional regulator	Rv3334	-	transcriptional regulator (MerR family)			
Rv1152	-	transcriptional regulator (GntR family)	Rv3405c	-	putative transcriptional regulator			
Rv1167c	-	putative transcriptional regulator	Rv3522	-	putative transcriptional regulator			
Rv1219c	-	putative transcriptional regulator	Rv3557c	-	transcriptional regulator (TetR/AcrR family)			
Rv1255c	-	transcriptional regulator (TetR/AcrR family)	Rv3574	-	transcriptional regulator (TetR/AcrR family)			
Rv1332	-	putative transcriptional regulator	Rv3575c	-	transcriptional regulator (LacI family)			
Rv1353c	-	transcriptional regulator (TetR/AcrR family)	Rv3583c	-	putative transcriptional regulator			
Rv1358	-	transcriptional regulator (LuxR/UhpA family)	Rv3676	-	transcriptional regulator (Crp/Fnr family)			
Rv1359	-	putative transcriptional regulator	Rv3678c	-	transcriptional regulator (LysR family)			
Rv1395	-	transcriptional regulator (AraC/XylS family)	Rv3736	-	transcriptional regulator (AraC/XylS family)			
Rv1404	-	transcriptional regulator (MarR family)	Rv3744	-	transcriptional regulator (ArsR family)			
Rv1423	-	putative transcriptional regulator	Rv3830c	-	transcriptional regulator (TetR/AcrR family)			
Rv1460	-	putative transcriptional regulator	Rv3833	-	transcriptional regulator (AraC/XylS family)			
Rv1474c	-	transcriptional regulator (TetR/AcrR family)	Rv3840	-	putative transcriptional regulator			
Rv1534	-	transcriptional regulator (TetR/AcrR family)	Rv3855	-	putative transcriptional regulator			
Rv1556	-	putative transcriptional regulator	2. Two component systems Rv1028c <i>kdpD</i> sensor histidine kinase Rv1027c <i>kdpE</i> two-component response regulator Rv3246c <i>mtrA</i> two-component response regulator Rv3245c <i>mtrB</i> sensor histidine kinase Rv0844c <i>narL</i> two-component response regulator Rv0757 <i>phoP</i> two-component response regulator Rv0758 <i>phoR</i> sensor histidine kinase Rv0491 <i>regX3</i> two-component response regulator Rv0490 <i>senX3</i> sensor histidine kinase Rv0602c <i>tcra</i> two-component response regulator Rv0260c - two-component response regulator Rv0600c - sensor histidine kinase Rv0601c - sensor histidine kinase Rv0818 - two-component response regulator Rv0845 - sensor histidine kinase Rv0902c - sensor histidine kinase Rv0903c - two-component response regulator Rv0981 - two-component response regulator Rv0982 - sensor histidine kinase Rv1032c - sensor histidine kinase Rv1033c - two-component response regulator Rv1626 - two-component response regulator Rv2027c - sensor histidine kinase Rv2884 - two-component response regulator Rv3132c - sensor histidine kinase Rv3133c - two-component response regulator Rv3143 - putative sensory transduction protein Rv3220c - sensor histidine kinase Rv3764c - sensor histidine kinase Rv3765c - two-component response regulator					
Rv1674c	-	putative transcriptional regulator						
Rv1675c	-	putative transcriptional regulator						
Rv1719	-	transcriptional regulator (lclR family)						
Rv1773c	-	transcriptional regulator (lclR family)						
Rv1776c	-	putative transcriptional regulator						
Rv1816	-	putative transcriptional regulator						
Rv1846c	-	putative transcriptional regulator						
Rv1931c	-	transcriptional regulator (AraC/XylS family)						
Rv1956	-	putative transcriptional regulator						
Rv1963c	-	putative transcriptional regulator						
Rv1985c	-	transcriptional regulator (LysR family)						
Rv1990c	-	putative transcriptional regulator						
Rv1994c	-	transcriptional regulator (MerR family)						
Rv2017	-	putative transcriptional regulator (PbsX/Xre family)						
Rv2021c	-	putative transcriptional regulator						
Rv2034	-	transcriptional regulator (ArsR family)						
Rv2175c	-	putative transcriptional regulator						
Rv2250c	-	putative transcriptional regulator						
Rv2258c	-	putative transcriptional regulator						
Rv2282c	-	transcriptional regulator (LysR family)						
Rv2308	-	putative transcriptional regulator						
Rv2324	-	transcriptional regulator (Lrp/AsnC family)						
Rv2358	-	transcriptional regulator (ArsR family)						
Rv2488c	-	transcriptional regulator (LuxR/UhpA family)						
Rv2506	-	transcriptional regulator (TetR/AcrR family)						
Rv2621c	-	putative transcriptional regulator						
Rv2640c	-	transcriptional regulator (ArsR family)						
Rv2642	-	transcriptional regulator (ArsR family)						
Rv2669	-	putative transcriptional regulator						
Rv2745c	-	putative transcriptional regulator						
Rv2779c	-	transcriptional regulator (Lrp/AsnC family)						
Rv2887	-	transcriptional regulator (MarR family)						
Rv2912c	-	transcriptional regulator (TetR/AcrR family)						
Rv2989	-	transcriptional regulator (lclR family)						
Rv3050c	-	putative transcriptional regulator						
Rv3055	-	putative transcriptional regulator						
Rv3058c	-	putative transcriptional regulator						
Rv3060c	-	transcriptional regulator (GntR family)						
Rv3066	-	putative transcriptional regulator						
Rv3095	-	putative transcriptional regulator						
Rv3124	-	transcriptional regulator (AfsR/DndI/RedD family)						
3. Serine-threonine protein kinases and phosphoprotein phosphatases Rv0015c <i>pknA</i> serine-threonine protein kinase Rv0014c <i>pknB</i> serine-threonine protein kinase Rv0931c <i>pknD</i> serine-threonine protein kinase Rv1743 <i>pknE</i> serine-threonine protein kinase Rv1746 <i>pknF</i> serine-threonine protein kinase Rv0410c <i>pknG</i> serine-threonine protein kinase Rv1266c <i>pknH</i> serine-threonine protein kinase Rv2914c <i>pknI</i> serine-threonine protein kinase Rv2088 <i>pknJ</i> serine-threonine protein kinase Rv3080c <i>pknK</i> serine-threonine protein kinase Rv2176 <i>pknL</i> serine-threonine protein kinase			2. Ribosome modification and maturation Rv1010 <i>ksgA</i> 16S rRNA dimethyltransferase Rv2838c <i>rbfA</i> ribosome-binding factor A Rv2907c <i>rimM</i> 16S rRNA processing protein 3. Aminoacyl tRNA synthetases and their modification Rv2555c <i>alaS</i> alanyl-tRNA synthase Rv1292 <i>argS</i> arginyl-tRNA synthase Rv2572c <i>aspS</i> aspartyl-tRNA synthase Rv3580c <i>cysS</i> cysteinyl-tRNA synthase Rv2130c <i>cysS2</i> cysteinyl-tRNA synthase Rv1406 <i>fnt</i> methionyl-tRNA formyltransferase Rv3011c <i>gatA</i> glu-tRNA-gln amidotransferase, subunit B Rv3009c <i>gatB</i> glu-tRNA-gln amidotransferase, subunit A Rv3012c <i>gatC</i> glu-tRNA-gln amidotransferase, subunit C Rv2992c <i>gltS</i> glutamyl-tRNA synthase Rv2357c <i>glyS</i> glycyl-tRNA synthase Rv2580c <i>hisS</i> histidyl-tRNA synthase Rv1536 <i>ileS</i> isoleucyl-tRNA synthase Rv0041 <i>leuS</i> leucyl-tRNA synthase Rv3598c <i>lysS</i> lysyl-tRNA synthase Rv1640c <i>lysX</i> C-term lysyl-tRNA synthase Rv1007c <i>metS</i> methionyl-tRNA synthase Rv1649 <i>pheS</i> phenylalanyl-tRNA synthase α subunit					

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Rv2715 - lase
2-hydroxymuconic semialdehyde
hydrolase
Rv3530c - probable *cis*-diol dehydrogenase
Rv3534c - 4-hydroxy-2-oxovalerate aldolase
Rv3536c - aromatic hydrocarbon degradation

C. Cell envelope

1. Lipoproteins (*lppA-lppO*) 65

2. Surface polysaccharides, lipopolysaccharides, proteins and antigens

Rv0806c *cpsY* probable UDP-glucose-4-epimerase
secreted protein
Rv3811 *csp* highly similar to C-term Mpt53
Rv1677 *dsbF* involved in arabinogalactan synthesis
Rv3794 *embA* involved in arabinogalactan synthesis
Rv3795 *embB* involved in arabinogalactan synthesis
Rv3793 *embC* involved in arabinogalactan synthesis
Rv3875 *esat6* early secretory antigen target
Rv0112 *gca* probable GDP-mannose dehydratase
Rv0113 *gmhA* phosphoheptose isomerase
Rv2965c *kdtB* lipopolysaccharide core biosynthesis protein
Rv2878c *mpt53* secreted protein Mpt53
Rv1980c *mpt64* secreted immunogenic protein Mpb64/Mpt64
Rv2875 *mpt70* major secreted immunogenic protein Mpt70 precursor
Rv2873 *mpt83* surface lipoprotein Mpt83
Rv0899 *ompA* member of OmpA family
Rv3810 *pirG* cell surface protein precursor (Erp protein)
Rv3782 *rfe* similar to rhamnosyl transferase
Rv1302 *rfe* undecaprenyl-phosphate α -N-acetylglucosaminyltransferase
Rv2145c *wag31* antigen 84 (aka wag31)
Rv0431 - tuberculin related peptide (AT103)
Rv0954 - cell envelope antigen
Rv1514c - involved in polysaccharide synthesis
Rv1518 - involved in exopolysaccharide synthesis
Rv1758 - partial cutinase
Rv1910c - probable secreted protein
Rv1919c - weak similarity to pollen antigens
Rv1984c - probable secreted protein
Rv1987 - probable secreted protein
Rv2223c - probable exported protease
Rv2224c - probable exported protease
Rv2301 - probable cutinase
Rv2345 - precursor of probable membrane protein
Rv2672 - putative exported protease
Rv3019c - similar to Esat6
Rv3036c - probable secreted protein
Rv3449 - probable precursor of serine protease
Rv3451 - probable cutinase
Rv3452 - probable cutinase precursor
Rv3724 - probable cutinase precursor

3. Murein sacculus and peptidoglycan

Rv2911 *dacB* penicillin binding protein
Rv2981c *dlaA* D-alanine-D-alanine ligase A
Rv3809c *glf* UDP-galactopyranose mutase
Rv1018c *glmU* UDP-N-acetylglucosamine pyrophosphorylase
Rv3382c *lytB1* LytB protein homologue
Rv1110 *lytB2* very similar to LytB
Rv1315 *murA* UDP-N-acetylglucosamine-1-carboxyvinyltransferase
Rv0482 *murB* UDP-N-acetylenolpyruvylglucosamine reductase
Rv2152c *murC* UDP-N-acetyl-muramate-alanine ligase
Rv2155c *murD* UDP-N-acetylmuramoylalanine-D-glutamate ligase
Rv2158c *murE* *meso*-diaminopimelate-adding enzyme
Rv2157c *murF* D-alanine:D-alanine-adding enzyme
Rv2153c *murG* transferase in peptidoglycan synthesis
Rv1338 *muri* glutamate racemase
Rv2156c *murX* phospho-N-acetylmuramoyl-petapetide transferase
Rv3332 *nagA* N-acetylglucosamine-6-P-deacetylase
Rv0016c *pbpA* penicillin-binding protein
Rv2163c *pbpB* penicillin-binding protein 2
Rv0050 *ponA1* penicillin-binding protein
Rv3682 *ponA2* class A penicillin binding protein
Rv0017c *rodA* FtsW/RodA/SpvE family
Rv0907 - probable penicillin binding protein

Rv1367c - probable penicillin binding protein
Rv1730c - probable penicillin binding protein
Rv1922 - probable penicillin binding protein
Rv2864c - probable penicillin binding protein
Rv3330 - probable penicillin binding protein
Rv3627c - probable penicillin binding protein

4. Conserved membrane proteins

Rv0402c *mmpL1* conserved large membrane protein
Rv0507 *mmpL2* conserved large membrane protein
Rv0206c *mmpL3* conserved large membrane protein
Rv0450c *mmpL4* conserved large membrane protein
Rv0676c *mmpL5* conserved large membrane protein
Rv1557 *mmpL6* conserved large membrane protein
Rv2942 *mmpL7* conserved large membrane protein
Rv3823c *mmpL8* conserved large membrane protein
Rv2339 *mmpL9* conserved large membrane protein
Rv1183 *mmpL10* conserved large membrane protein
Rv0202c *mmpL11* conserved large membrane protein
Rv1522c *mmpL12* conserved large membrane protein
Rv0403c *mmpS1* conserved small membrane protein
Rv0506 *mmpS2* conserved small membrane protein
Rv2198c *mmpS3* conserved small membrane protein
Rv0451c *mmpS4* conserved small membrane protein
Rv0677c *mmpS5* conserved small membrane protein

5. Other membrane proteins 211

III. Cell processes

A. Transport/binding proteins

1. Amino acids
Rv2127 *ansP* L-asparagine permease
Rv0346c *aroP2* probable aromatic amino acid permease
Rv0917 *betP* glycine betaine transport
Rv1704c *cycA* transport of D-alanine, D-serine and glycine
Rv3666c *dppA* probable peptide transport system permease
Rv3665c *dppB* probable peptide transport system permease
Rv3664c *dppC* probable peptide transport system permease
Rv3663c *dppD* probable ABC-transporter
Rv0522 *gabP* probable 4-amino butyrate transporter
Rv0411c *glnH* putative glutamine binding protein
Rv2564 *glnQ* probable ATP-binding transport protein
Rv1280c *oppA* probable oligopeptide transport protein
Rv1283c *oppB* oligopeptide transport protein
Rv1282c *oppC* oligopeptide transport system permease
Rv1281c *oppD* probable peptide transport protein
Rv2320c *rocE* arginine/ornithine transporter
Rv3253c - probable cationic amino acid transport
Rv3454 - possible proline permease

2. Cations

Rv2920c *amt* putative ammonium transporter
Rv1607 *chaA* putative calcium/proton antiporter
Rv1239c *corA* probable magnesium and cobalt transport protein
Rv0092 *ctpA* cation-transporting ATPase
Rv0103c *ctpB* cation transport ATPase
Rv3270 *ctpC* cation transport ATPase
Rv1469 *ctpD* probable cadmium-transporting ATPase
Rv0908 *ctpE* probable cation transport ATPase
Rv1997 *ctpF* probable cation transport ATPase
Rv1992c *ctpG* probable cation transport ATPase
Rv0425c *ctpH* C-terminal region putative cation-transporting ATPase
Rv0107c *ctpl* probable magnesium transport ATPase
Rv0969 *ctpV* cation transport ATPase
Rv3044 *fecB* putative FeIII-dicitrate transporter
Rv0265c *fecB2* iron transport protein FeIII dicitrate transporter
Rv1029 *kdpA* potassium-transporting ATPase A chain

Rv1030 *kdpB* potassium-transporting ATPase B chain
Rv1031 *kdpC* potassium-transporting ATPase C chain
Rv3236c *kefB* probable glutathione-regulated potassium-efflux protein
Rv2877c *merT* possible mercury resistance transport system
Rv1811 *mgtC* probable magnesium transport ATPase protein C
Rv0362 *mgtE* putative magnesium ion transporter
Rv2856 *nicT* probable nickel transport protein
Rv0924c *nramp* transmembrane protein belonging to Nramp family
Rv2691 *trkA* probable potassium uptake protein
Rv2692 *trkB* probable potassium uptake protein
Rv2287 *yjcE* probable Na⁺/H⁺ exchanger
Rv2723 - probable membrane protein, tellurium resistance
Rv3162c - probable membrane protein
Rv3237c - possible potassium channel protein
Rv3743c - probable cation-transporting ATPase

3. Carbohydrates, organic acids and alcohols

Rv2443 *dctA* C4-dicarboxylate transport protein
Rv3476c *kgtP* sugar transport protein
Rv1902c *nanT* probable sialic acid transporter
Rv1236 *sugA* membrane protein probably involved in sugar transport
Rv1237 *sugB* sugar transport protein
Rv1238 *sugC* ABC transporter component of sugar uptake system
Rv3331 *sugI* probable sugar transport protein
Rv2835c *ugpA* *sn*-glycerol-3-phosphate permease
Rv2833c *ugpB* *sn*-glycerol-3-phosphate-binding periplasmic lipoprotein
Rv2832c *ugpC* *sn*-glycerol-3-phosphate transport ATP-binding protein
Rv2834c *ugpE* *sn*-glycerol-3-phosphate transport system protein
Rv2316 *uspA* sugar transport protein
Rv2318 *uspC* sugar transport protein
Rv2317 *uspE* sugar transport protein
Rv1200 - probable sugar transporter
Rv2038c - probable ABC sugar transporter
Rv2039c - probable sugar transporter
Rv2040c - probable sugar transporter
Rv2041c - probable sugar transporter

4. Anions

Rv2684 *arsA* probable arsenical pump
Rv2685 *arsB* probable arsenical pump
Rv3578 *arsB2* probable arsenical pump
Rv2643 *arsC* probable arsenical pump
Rv2397c *cysA* sulphate transport ATP-binding protein
Rv2399c *cysT* sulphate transport system permease protein
Rv2398c *cysW* sulphate transport system permease protein
Rv1857 *modA* molybdate binding protein
Rv1858 *modB* transport system permease, molybdate uptake
Rv1859 *modC* molybdate uptake ABC-transporter
Rv1860 *modD* precursor of Apa (45/47 kD secreted protein)
Rv2329c *narK1* probable nitrite extrusion protein
Rv1737c *narK2* nitrite extrusion protein
Rv0261c *narK3* nitrite extrusion protein
Rv0267 *narU* similar to nitrite extrusion protein 2
Rv0934 *phoS1* PstS component of phosphate uptake
Rv0928 *phoS2* PstS component of phosphate uptake
Rv0820 *phoT* phosphate transport system ABC transporter
Rv3301c *phoY1* phosphate transport system regulator
Rv0821c *phoY2* phosphate transport system regulator
Rv0545c *pitA* low-affinity inorganic phosphate transporter
Rv2281 *pitB* phosphate permease
Rv0930 *pstA1* PstA component of phosphate uptake
Rv0936 *pstA2* PstA component of phosphate uptake
Rv0933 *pstB* ABC transport component of phosphate uptake
Rv0935 *pstC* PstC component of phosphate uptake
Rv0929 *pstC2* membrane-bound component of

Rv0932c *pstS* phosphate transport system
PstS component of phosphate uptake
Rv2400c *subI* sulphate binding precursor
Rv0143c - probable chloride channel
Rv1707 - probable sulphate permease
Rv1739c - possible sulphate transporter
Rv3679 - possible anion transporter
Rv3680 - probable anion transporter

5. Fatty acid transport
Rv2790c *ltp1* non-specific lipid transport protein
Rv3540c *ltp2* non-specific lipid transport protein

6. Efflux proteins
Rv2936 *drfA* similar daunorubicin resistance ABC-transporter
Rv2937 *drfB* similar daunorubicin resistance transmembrane protein
Rv2938 *drfC* similar daunorubicin resistance transmembrane protein
Rv2846c *efpA* putative efflux protein
Rv3065 *emrE* resistance to ethidium bromide
Rv0783c - multidrug resistance protein
Rv0849 - possible quinolone efflux pump
Rv1145 - probable drug transporter
Rv1146 - probable drug transporter
Rv1250 - probable drug efflux protein
Rv1258c - probable multidrug resistance pump
Rv1410c - probable drug efflux protein
Rv1634 - probable drug efflux protein
Rv1819c - probable multidrug resistance pump
Rv2136c - putative bacitracin resistance protein
Rv2209 - probable drug efflux protein
Rv2333c - probable tetracycline C resistance protein
Rv2994 - probable fluoroquinolone efflux protein
Rv1877 - probable drug efflux protein
Rv2459 - probable drug efflux protein

B. Chaperones/Heat shock
Rv0384c *clpB* heat shock protein
Rv0352 *dnaJ* acts with GrpE to stimulate DnaK ATPase
Rv2373c *dnaJ2* DnaJ homologue
Rv0350 *dnaK* 70 kD heat shock protein, chromosome replication
Rv3417c *groEL1* 60 kD chaperonin 1
Rv0440 *groEL2* 60 kD chaperonin 2
Rv3418c *groES* 10 kD chaperone
Rv0351 *grpE* stimulates DnaK ATPase activity
Rv2374c *hrcA* heat-inducible transcription repressor
Rv0251c *hsp* possible heat shock protein
Rv0353 *hspR* heat shock regulator
Rv2031c *hspX* 14kD antigen, heat shock protein Hsp20 family
Rv2299c *htpG* heat shock protein Hsp90 family
Rv0563 *htpX* probable (transmembrane) heat shock protein
Rv2701c *subB* putative extragenic suppressor protein
Rv3269 - probable heat shock protein

C. Cell division
Rv3641c *fic* possible cell division protein
Rv3102c *ftsE* membrane protein
Rv3610c *ftsH* inner membrane protein, chaperone
Rv2748c *ftsK* chromosome partitioning
Rv2151c *ftsQ* ingrowth of wall at septum
Rv2154c *ftsW* membrane protein (shape determination)
Rv3101c *ftsX* membrane protein
Rv2921c *ftsY* cell division protein FtsY
Rv2150c *ftsZ* circumferential ring, GTPase
Rv3919c *gid* glucose inhibited division protein B
Rv3625c *mesJ* probable cell cycle protein
Rv3917c *parA* chromosome partitioning; DNA-binding
Rv3918c *parB* possibly involved in chromosome partitioning
Rv2922c *smc* member of Smc1/Cut3/Cut14 family
Rv0012 - possible cell division protein
Rv0435c - ATPase of AAA-family
Rv2115c - ATPase of AAA-family
Rv3213c - possible role in chromosome segregation
Rv1708 - possible role in chromosome partitioning

D. Protein and peptide secretion
Rv2916c *ffh* signal recognition particle protein
Rv2903c *lepB* signal peptidase I
Rv1614 *lgt* prolipoprotein diacylglycerol transferase
Rv1539 *lspA* lipoprotein signal peptidase
Rv0379 *sec* probable transport protein SecE/Sec61-γ family
Rv3240c *secA* SecA, preprotein translocase sub-

Rv1821 *secA2* unit
SecA, preprotein translocase sub-unit
Rv2587c *secD* protein-export membrane protein
Rv0638 *secE* SecE preprotein translocase
Rv2586c *secF* protein-export membrane protein
Rv1440 *secG* protein-export membrane protein
Rv0732 *secY* SecY subunit of preprotein translocase
Rv2462c *tig* chaperone protein, similar to trigger factor
Rv2813 - probable general secretion pathway protein

E. Adaptations and atypical conditions
Rv1901 *cinA* competence damage protein
Rv3648c *cspA* cold shock protein, transcriptional regulator
Rv0871 *cspB* probable cold shock protein
Rv3063 *cstA* starvation-induced stress response protein
Rv3490 *otsA* probable α,α-trehalose-phosphate synthase
Rv2006 *otsB* trehalose-6-phosphate phosphatase
Rv3372 *otsB2* trehalose-6-phosphate phosphatase
Rv3758c *proV* osmoprotection ABC transporter
Rv3757c *proW* transport system permease
Rv3759c *proX* similar to osmoprotection proteins
Rv3756c *proZ* transport system permease
Rv1026 - probable pppGpp-5'phosphohydrolyase

F. Detoxification
Rv2428 *ahpC* alkyl hydroperoxide reductase
Rv2429 *ahpD* member of AhpC/TSA family
Rv2238c *ahpE* member of AhpC/TSA family
Rv2521 *bcp* bacterioferritin comigratory protein
Rv1608c *bcpB* probable bacterioferritin comigratory protein
Rv3473c *bpoA* probable non-heme bromoperoxidase
Rv1123c *bpoB* probable non-heme bromoperoxidase
Rv0554 *bpoC* probable non-heme bromoperoxidase
Rv3617 *ephA* probable epoxide hydrolase
Rv1938 *ephB* probable epoxide hydrolase
Rv1124 *ephC* probable epoxide hydrolase
Rv2214c *ephD* probable epoxide hydrolase
Rv3670 *ephE* probable epoxide hydrolase
Rv0134 *ephF* probable epoxide hydrolase
Rv3171c *hpx* probable non-heme haloperoxidase
Rv1908c *katG* catalase-peroxidase
Rv3846 *sodA* superoxide dismutase
Rv0432 *sodC* superoxide dismutase precursor - (Cu-Zn)
Rv1932 *tpx* thiol peroxidase
Rv0634c - putative glyoxylase II
Rv2581c - putative glyoxylase II
Rv3177 - probable non-heme haloperoxidase

IV. Other
A. Virulence
Rv0169 *mce1* cell invasion protein
Rv0589 *mce2* cell invasion protein
Rv1966 *mce3* cell invasion protein
Rv3499c *mce4* cell invasion protein
Rv3100c *smfB* probable small protein b
Rv1694 *tylA* cytotoxin/hemolysin homologue
Rv0024 - putative p60 homologue
Rv0167 - part of *mce1* operon
Rv0168 - part of *mce1* operon
Rv0170 - part of *mce1* operon
Rv0171 - part of *mce1* operon
Rv0172 - part of *mce1* operon
Rv0174 - part of *mce1* operon
Rv0587 - part of *mce2* operon
Rv0588 - part of *mce2* operon
Rv0590 - part of *mce2* operon
Rv0591 - part of *mce2* operon
Rv0592 - part of *mce2* operon
Rv0594 - part of *mce2* operon
Rv1085c - possible hemolysin
Rv1477 - putative exported p60 protein homologue
Rv1478 - putative exported p60 protein homologue
Rv1566c - putative exported p60 protein homologue
Rv1964 - part of *mce3* operon
Rv1965 - part of *mce3* operon
Rv1967 - part of *mce3* operon
Rv1968 - part of *mce3* operon
Rv1969 - part of *mce3* operon
Rv1971 - part of *mce3* operon
Rv2190c - putative p60 homologue
Rv3494c - part of *mce4* operon
Rv3496c - part of *mce4* operon
Rv3497c - part of *mce4* operon
Rv3498c - part of *mce4* operon

Rv3500c - part of *mce4* operon
Rv3501c - part of *mce4* operon
Rv3896c - putative p60 homologue
Rv3922c - possible hemolysin

B. IS elements, Repeated sequences, and Phage
1. IS elements
IS6110 16 copies
IS1081 6 copies
Others 34 copies

2. REP13E12 family 7 copies

3. Phage-related functions
Rv2894c *xerC* integrase/recombinase
Rv1701 *xerD* integrase/recombinase
Rv1054 - integrase-a
Rv1055 - integrase-b
Rv1573 - phiRV1 phage related protein
Rv1574 - phiRV1 phage related protein
Rv1575 - phiRV1 phage related protein
Rv1576c - phiRV1 phage related protein
Rv1577c - phiRV1 possible prohead protease
Rv1578c - phiRV1 phage related protein
Rv1579c - phiRV1 phage related protein
Rv1580c - phiRV1 phage related protein
Rv1581c - phiRV1 phage related protein
Rv1582c - phiRV1 phage related protein
Rv1583c - phiRV1 phage related protein
Rv1584c - phiRV1 phage related protein
Rv1585c - phiRV1 phage related protein
Rv1586c - phiRV1 integrase
Rv2309c - integrase
Rv2310 - excisionase
Rv2646 - phiRV2 integrase
Rv2647 - phiRV2 phage related protein
Rv2650c - phiRV2 phage related protein
Rv2651c - phiRV2 prohead protease
Rv2652c - phiRV2 phage related protein
Rv2653c - phiRV2 phage related protein
Rv2654c - phiRV2 phage related protein
Rv2655c - phiRV2 phage related protein
Rv2656c - phiRV2 phage related protein
Rv2657c - similar to gp36 of mycobacteriophage L5
Rv2658c - phiRV2 phage related protein
Rv2659c - phiRV2 integrase
Rv2830c - similar to phage P1 *phd* gene
Rv3750c - excisionase
Rv3751 - putative integrase

C. PE and PPE families
1. PE family
PE subfamily 38 members
PE_PGRS subfamily 61 members

2. PPE family 68 members

D. Antibiotic production and resistance
Rv2068c *blaC* class A β-lactamase
Rv3290c *lat* lysine-ε aminotransferase
Rv2043c *pncA* pyrazinamide resistance/sensitivity
Rv0133 - possible puromycin N-acetyltransferase
Rv0262c - aminoglycoside 2'-N-acetyltransferase
Rv0802c - acetyltransferase
Rv1082 - similar to *S. lincolnensis* *ImbE*
Rv1170 - similar to *S. lincolnensis* *ImbE*
Rv1347c - possible aminoglycoside 6'-N-acetyltransferase
Rv2036 - similar to lincomycin production genes
Rv2303c - similar to *S. griseus* macrotetrolide resistance protein
Rv3225c - probable aminoglycoside 3'-phosphotransferases
Rv3700c - probable acetyltransferase
Rv3817 - probable aminoglycoside 3'-phosphotransferase

E. Bacteriocin-like proteins 3

F. Cytochrome P450 enzymes 22

G. Coenzyme F420-dependent enzymes 3

H. Miscellaneous transferases 61

I. Miscellaneous phosphatases, lyases, and hydrolases 18

J. Cyclases 6

K. Chelataes 2

V. Conserved hypotheticals 912

VI. Unknowns 606

TOTAL 3924